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A Comparison of Two Markers of Cell Proliferation in Bone Marrow*

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The cell cycle, or time between successive mitoses, can be structured into several phases based on studies with two markers precursor incorporation into DNA and the process of mitosis itself (6) In rapidly dividing cell systems such as intestinal epithelium and bone marrow the synthesis of new DNA is thought to be associated almost entirely with the preparations for mitosis. On the other hand, in slowly or rarely dividing tissues, the incorporation of precursors into DNA may be considerably greater than anticipated from the degree of mitotic activity (13) this difference is presumed to represent metabolic turnover In this paper we wish to present recent data on mitotic activity of bone marrow in dog and man, and to compare proliferation rates based on this marker with rates derived from labeling with tritiated thymidine.

Materials and Methods

Six beagles, 3 male and 3 female, were chosen for study of mitotic indices. Bone marrow samples were aspirated, under local anesthesia, from the proximal end of the femur into heparinized dog plasma (approximately 0.5 ml marrow in 0.5 ml plasma). About two-thirds of the sample was immediately transferred to centrifuge tube containing 3 to 5 ml of 1 12% sodium citrate solution at 37 C; the remainder was used immediately to prepare smears (Wright stain). After 5 minutes incubation with citrate the marrow suspension was centrifuged at 1500 rpm for 10 minutes. Nucleated cells, essentially those in the buffy coat, were then transferred to tube containing 12 ml of freshly prepared fixative (glycerine glacial acetic acid absolute ethyl alcohol 1 3 9 by volume) The contents were agitated gently to break any clumps. After 30 minutes fixation, the suspension was centrifuged at 1000 rpm for 10 minutes. About 0.5 ml of freshly prepared glacial acetic acid-absolute ethyl alcohol (1 3 by volume) was added to the cells, which were uniformly suspended by repeated pipetting. Smears were made by placing drop of the cell suspension on an inclined chilled slide (9). After drying,

* This work was performed under the auspices of the U S. Atomic Energy Commission.

the smears were immersed in absolute ethyl alcohol for 15 minutes and subsequently stained with aceto-orcein (15)

In the studies with man, marrow samples were aspirated from the sternum into heparinized human plasma. The time of sampling (9 a. m. to 2 p. m.) and all other procedures were otherwise identical to those described for the dog. Marrow samples were obtained from 8 hematologically normal male patients through the cooperation of Dr ALBERTO MOORE of the Hektoen Institute, Cook County Hospital, Chicago.

Total mitotic counts in the marrow sample were obtained from the aceto-orcein preparation; differential mitotic and cell counts from the Wright-stained smears. A minimum of 5000 nucleated cells, exclusive of segmented cells, was scored for mitoses under oil immersion objective on both the orcein and Wright-stained preparations. For differential counting, 1000 myeloid cells and the associated nucleated erythroid cells were enumerated. All samples of dog bone marrow and half of the human marrow preparations were evaluated independently by two observers, one of whom counted at least 1000 cells and the other 5000 cells on different smears of the same marrow aspirate. The mean mitotic indices (mitoses per 100 cells) of total marrow in the dog were 4.4 ± 0.2 and 5.0 ± 0.2 for the two observers In the 4 patients where independant observations were made, the mean mitotic indices were 4.2 ± 0.4 and 4.3 ± 0.4 Since there were no significant differences in the various mitotic counts and in the differential counts, the data were combined for presentation in the tables.

Results

The proportional distribution of nucleated cells in bone marrow aspirates of man and dog, as observed in Wright-stained smears, is shown in table I. Proliferative erythroid cells include

Table I

Proportional distribution of nucleated cells in bone marrow aspirates (mean and standard error).

		Myeloid							Erythroid		Myeloid* erythroid ratio
Species	No. of subj.	Mb	PM	My	MM	B	S	L	PC	NPC	
Dog	6	2.[illegible]	2.8	30.0	19.3	26.3	13.2	6.3	43.0	33.8	1.14
		0.3	0.4	1.8	1.1	2.0	0.8	0.5	5.5	2.9	0.11
Man	8	2.1	2.6	36.0	16.1	17.9	17.5	7.8	21.4	18.5	1.94
		0.2	0.2	1.5	1.2	1.4	2.4	1.0	0.6	1.7	0.15

Mb = Myeloblast PM = promyelocyte My = myelocyte, MM = metamyelocyte B = band cell S = segmented cell L = lymphocyte PC = proliferating cells NPC = non-proliferating cells.

* Exclusive of segmented cells and lymphocytes.

erythroblasts, basophilic normoblasts and most of the polychromatic normoblasts that label initially with H^3 thymidine. Similarly myelocytes include both the large and small variety even with some degree of nuclear indentation such cells incorporate H^3 thymidine. The distribution of mitotic stages and the percentage

Table II

Phase distribution of mitoses in orcein preparations of marrow (mean and standard error per 100 nucleated cells exclusive of segmented cells)

Species	Number Subjects	Prophase	Metaphase	Anaphase	Telophase	Total
Dog	6	2.5±0.1	1.1±0.04	0.3±0.04	0.8±0.06	4.7±0.2
Man	8	2.3±0.2	1.0±0.06	0.3±0.02	0.7±0.1	4.3±0.3

of mitoses as seen in orcein preparations are presented in table II When nuclear chromatin was elongated and appeared as dark staining loose threads, the cells were scored as early prophases. Since chromatin tends to be coarser in erythroid nuclei, the distinction between the interphase nucleus and early prophase is somewhat more difficult than in myeloid cells. When the nuclear membrane disappeared, cells were considered to be in late prophase. Cells were scored as metaphases when individual chromosomes were paired when chromosomes moved apart from the equatorial plate, cells were in anaphase. Cells were classified as telophases when the cytoplasm was partly or almost completely divided with the chromosomes still distinct. However the chromatin at opposite poles may be quite organized in marrow cells before fission of the cytoplasm and such cells were also scored as telophases. As may be noted in table II the phase distribution of mitoses is similar in man and dog Prophases were about twice as numerous as metaphases.

The total marrow mitotic index in the panchromatic smear ranged from 60 to 94 % of the mitotic index in the aceto-orcein preparation (average, 76 % in dog and 85 % in man) metaphase and anaphase counts were in good agreement in the two stained preparations, the Wright stain values being 94–100 % of the orcein values. With the panchromatic stain, prophases were more difficult to recognize in both myeloid and erythroid cells until the nuclear membrane was lost. Metaphase *chromosomes were conspicuous* when they lay flat in rosettes in end-on view they appeared as thick, corrugated, bar-like nuclei, which might be interpreted to represent more mature myeloid cells. Anaphases were easy to recognize in either case. With the Wright-stained smear there were fewer myeloid than erythroid telophases in proportion to metaphases, perhaps because of a greater susceptibility of the former to breakage during smearing

Tables III A and B present the specific mitotic indices for different marrow cell populations in dog and man based on the

Table III A
Mitotic indices (canine bone marrow)

Subject	Age mos.	Mitoses per 100 cells					Mitoses myeloid erythroid
		Total*	Myeloid P.C.	Myelocyte	Erythroid Total	P.C. **	
79	52	4.2	12.9	11.6	7.0	12.7	46.54
88	38	4 8	10.3	9.8	4 7	8.0	45:55
27	41	3.3	8.0	7.3	6.4	12.5	4 .58
A43B	37	4.2	10.1	8.6	5.4	10 8	45:55
A43E	37	4 1	9.3	8.1	4.5	7 4	49.51
A47A	34	4.8	10.1	9.5	5.3	9 7	49.51
Mean		4.2	10.1	9.2	5.6	10.2	46.54
σ⁻		0.2	0.7	0.6	0.4	0.9	1 1:1 1

Exclusive of segmented cells and lymphocytes
Proliferative cells (myeloblast–myelocyt)
Proliferative cells (erythroblast–early polychromatic normoblast)

Table III B
Mitotic indices (human bone marrow).

Subject	Age, years	Mitoses per 100 cells					Mitoses myeloid erythroid
		Total	Myeloid P.C.	Myelocyte	Erythroid Total	P.C.	
E. J	47	3.2	6.0	5.4	6.8	12.5	48:52
C. W	7?	2.6	4 6	4 1	4.9	9 0	48:52
C. B.	22	3.8	6.5	5.4	8.8	16.8	45:55
H. K.	63	3.7	6.8	6.3	7.9	14 7	46.54
W. S.	61	3 0	4.5	4 0	4 7	9.2	46.54
S. W	57	4.0	8.0	7 1	10.2	15.7	51-49
H. W	54	2.9	5.5	4.8	7.5	12.2	49.51
H. S.	55	2.4	5.1	4.5	4.3	8 8	50:50
Mean		3.2	5.9	5.2	6.9	12.4	48:52
σ⁻		0.2	0.4	0.4	0.8	1 1	0.7:0.7

Exclusive of segmented cells and lymphocytes
Proliferative cells (myeloblast–myelocyt)
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total index derived from the orcein preparation and the differential mitotic count from the panchromatic smear The significance of the various indices and the possible relationship of these measurements to other parameters of the cell cycle will now be discussed.

Discussion

Mitotic indices for canine and human marrow observed in the present study are considerably greater than most reported values. We have no definite explanation for this other than to suggest that the nature of the measurement is such that one might anticipate

more false negatives than false positives. When prophases and telophases are excluded, it is of interest to note that the mitotic index for proliferative erythroid cells *in the dog is 3/*‰ *which is in ex*cellent agreement with the value reported by ALPEN AND CRANMORE (1) In addition to the problem of recognizing mitoses, to which we have already alluded, technical factors in preparation of marrow samples are no doubt contributory There is reason to believe that mitotic cells are more vulnerable to breakage and loss than interphase cells. If the cell membrane is not intact, particularly in the orcein preparation, proportionately fewer mitotic figures are seen. We have also noted that the number of mitoses decreases sharply when incubation of the marrow suspension with hypotonic citrate is delayed after marrow aspiration. Thus, as well as the *duration of incubation with citrate in the orcein preparation* and the time elapsing before smearing in the panchromatic preparation, may represent important variables.

The mitotic index, like other indices related to proliferative activity does not have a perspicuous meaning unless it can be related to a time constant. The value that is assigned to this parameter apparently depends upon the method of estimation as well as upon the stages of mitosis that are included. Periods ranging from 0.5 to 2.5 hours have been reported for various cell types in vertebrate tissues (14) Recent studies by ODARTCHENKO et al (10) who followed the rate of appearance of labeled mitoses after in*jection of tritiated thymidine*, are *suggestive of a mitotic duration* of 1 to 1.5 hours for canine erythroid cells. The metaphase time was estimated to be 20 minutes a similar value was reported previously by DUSTIN (4) from stathmokinetic analysis of rat bone marrow cells. If the duration of metaphase is taken as 20 minutes, the time for the entire mitotic process, based on the phase distribution (table II) would be about 1 4 hours for bone marrow cells in man and dog

The mitotic index (MI) for one or another component of the marrow cell population and the mitotic time (t_m) provide a measure of the proliferation rate, i. e. percent cells born per unit time, $k_p = \frac{MI}{t_m}$ Proliferation rates derived in this way should agree with those determined from the flash labeling index with tritiated thymidine (LI) if such labeled cells are destined to divide. Tritiated thymidine is believed to be incorporated selectively into cells that

are synthesizing new DNA and k_p should be given by $\frac{LI}{t_s}$. The more recent studies indicate that the DNA synthetic period (t_s) is reasonably constant and of the order of 5 to 7.5 hours in a variety of mammalian cells: [illegible] for granulocyte precursors in the dog is about 5 hours (8, 11); for erythroid cells a value of about 7 hours has been reported (10).

In the comparison of birth rates based upon the two markers of proliferative activity, i.e. mitosis and DNA synthesis, we have used t_m values of 1 and 1.5 hours and t_s values of 5 and 7.5 hours; these ranges would appear to embrace the uncertainty. The results are shown in table IV, where mitotic indices of the present study and those reported recently by KILLMANN et al. (7) for man are related to tritiated thymidine labeling indices from different labor-

Table IV

Comparison of proliferation rates derived from mitotic index and tritiated thymidine labeling

Proliferating cells	Proliferation rate, percent per hour			
	Mitotic index		³H-thymidine labeling index	
	t_m = 1 h	t_m = 1.5 h	t_s = 5 h	t_s = 7.5 h
Dog				
Granulocyte	10.1	6.7	8.0	5.3
Erythrocyte	10.2	6.7	11.4	7.6
Erythrocyte			14.0	9.3
Man				
Granulocyte	9	3.9	7	4.8
Granulocyte	1.2	0.8	7.0	4.7
Erythrocyte	1.4	2.3	1 0	8.0
Erythrocyte	0	3.3	10.2	6.7

Present [illegible] P [illegible] Ma [illegible]
O [illegible] et al. [illegible]
[illegible] Cronkite [illegible] et al. [illegible] 3, 5, 7

atories (2, 3, 10, 12). It may be noted first that while the latter are quite similar, this is not the case for the mitotic indices. The proliferation rates for human granulocyte and erythrocyte precursors based on the labeling indices are not in agreement with those derived from the mitotic indices of KILLMANN et al. The inconsistency is greater for granulocytes, a factor of 6, than for erythrocytes, a factor of 2, which for reasons described earlier may be a reflection of the greater tendency to underestimate granulocyte mitoses. On the other hand, the birth rates calculated from these two markers of proliferative activity of human marrow over

lap when the mitotic indices determined in the present study are used This applies also to the granulocyte and erythrocyte precursors in canine marrow (table IV) The agreement between the two markers holds reasonably well whether one considers the over all mitotic and labeling indices or the specific indices for a given cell type. *For example, k_p for dog myelocytes is 4.9 to 7 4 / per hour* from H^3 thymidine labeling (12) and 6 1 to 9 2 / per hour from the mitotic index. Similarly k_p for human myelocytes is 4.5 to 6.8 / per hour from labeling data (3 5) and 3.5 to 5.2 / per hour from mitotic data.

Summary

Mitotic indices were determined for different cell types in myeloid and erythroid populations of canine and human marrow Dividing cells in all phases were scored in orcein-stained marrow preparations. Specific mitotic indices were obtained from the total mitotic index of the orcein preparation and the differential cell and mitotic counts of marrow smears stained by Wright's method. Proliferation rates of myeloid and erythroid cells based on mitotic activity were related to rates determined from labeling with tritiated thymidine. There was reasonably good agreement between these two markers of cell proliferation, viz. mitosis and DNA synthesis.

Résumé

Les auteurs déterminaient l'index mitotique de divers types cellulaires de la population myéloique et érythropoétique de la moelle humaine et canine. On compte toutes les phases de la mitose sur le frottis coloré par l'orcéine. L index mitotique spécifique est calculé d'après l'index total du frottis coloré à l'orcéine et d'après le nombre de mitoses et la différentiation des cellules de frottis de la moelle, colorés selon la méthode de Wright. Puis les auteurs mettent la proportion des cellules de la série myéloique et erythropoétique en prolifération en relation avec les résultats obtenus par le marquage par la thymidine-tritium et obtiennent une bonne corrélation entre ces deux critères de la prolifération cellulaire, c'est-à-dire de la mitose et de la synthèse de l'ADN.

Zusammenfassung

Von den verschiedenen Zelltypen der myeloischen und erythropoetischen Population im Knochenmark von Hund und Mensch wurde der mitotische Index bestimmt. In Orcein-gefärbten Markpräparaten wurden alle Teilungsphasen ausgezählt. Die spezifischen mitotischen Indices wurden berechnet aus dem Gesamt-Mitoseindex des Orcein-Präparates und aus der Differentialzählung von Zellen und Mitosen in Knochenmarkausstrichen, die nach Wright gefärbt worden waren. Die Proliferationsraten myeloischer und erythroider Zellen auf Grund der Mitoseaktivität wurden in Beziehung gesetzt zu den Ergebnissen der Markierung mit Tritium-Thymidin. Dabei ergab sich eine ziemlich gute Übereinstimmung zwischen diesen beiden Kriterien der Zellproliferation, nämlich der Mitose und der DNA-Synthese.

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Istituto di Clinica Medica Generale e Terapia Medica dell'Università di Perugia
(Direttore: Prof. P. Larizza)

Electron Microscopic Observations on Bone Marrow and Liver Tissue in Non Hereditary Refractory Sideroblastic Anaemia

By Paolo Larizza and Francesco Orlandi

Sideroblastosis represents the fundamental element common to all hypochromic hypersideraemic anaemias (Heilmeyer's sideroachrestic anaemias). In these diseases a striking erythroblastic hyperplasia of the bone marrow is seen. The reticular cells and a great part of the erythroblasts are overcharged with iron. Ringed sideroblasts are frequent. The siderocytes are numerous. The granuloblastic elements are instead unaltered.

The accumulation of iron in the erythroblasts is provoked by a deficient utilisation caused by enzymatic defects in the porphyrin synthesis. These defects can occur at different levels along the metabolic chain that leads to the synthesis of haeme, that is: a) at the level of haeme synthetase, so that the incorporation of iron in the protoporphyrin is impeded and the erythrocytic protoporphyrin increases; b) at the level of the formation of the protoporphyrin from coproporphyrin, therefore decreasing the former and increasing the latter substance in the red blood cells; c) at the level of the conversion from porphobilinogen to urobilinogen; d) at the level of the first stages of the porphyrinogenesis, for example if the synthesis of the Δ-amino-laevulic acid is impeded; e) at two or more of the above mentioned levels.

Up to now electron microscopic observations of the bone marrow concerning six cases of idiophatic sideroachrestic anaemia have been published (1–6). We have observed under optic and electron microscopes the bone marrow and the hepatic tissue of a patient suffering from an idiopathic, non hereditary form of sideroachrestic anaemia, with increased erythrocytic protoporphyrin.

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Up to now electron microscopic observations of the bone marrow concerning six cases of idiophatic sideroachrestic anaemia have been published (1–6). We have observed under optic and electron microscopes the bone marrow and the hepatic tissue of a patient suffering from an idiopathic, non hereditary form of sideroachrestic anaemia, with increased erythrocytic protoporphyrin.

The clinical characteristics of the case were described in another article (7). Here we refer only to morphologic observations.

Method

Bone marrow. Small fragments were fixed in ... buffered ... from ... dehydrated ... embedded in Epon. The sections were cut ... Porter-Blum ultramicrotome and examined with Siemens Elmiskop I. The Perls reaction (9) was made on grids from each ... Semi-thin sections were also ... examined under the optic microscope.

Small fragments of the liver. The tissue ... small blocks of 1 mm diameter. They were fixed, embedded and examined ... the same method ... described for the bone marrow. A second part of the hepatic tissue was fixed in formol ... embedded as usual in paraffin. The usually employed staining techniques were used ... the Perls and the periodic acid-Schiff reaction made.

Results

Bone marrow. Under the optical microscope an intense erythroblastic hyperplasia is evident with a prevalence of basophilic forms. *The normoblastic characteristics of the tissue are almost completely* conserved. Some macroblasts are evident. Sixty per cent of the erythroblasts are overcharged with iron. The ringed sideroblasts are frequent. Numerous siderocytes are seen. The reticular cells are overcharged with iron. The white cells are morphologically normal.

Under the electron microscope numerous ultrastructural alterations are evident:

1. The cytoplasm of all the *reticular cells* is overcharged with iron which is present in different forms of aggregation as *D... ... m...ecules of ferritin*. They are well recognizable by their high opacity, their dimensions of about 50 Å and their morphology. Their aspect can be varied according to the plane of section but it is always referable to the well known octahedral model (10, 1?). In the reticular cells the dispersed ferritin molecules are very numerous in the whole cytoplasm (fig. ?) ... b... *aggregates of ferritin* ... The molecular structure of ferritin is well distinguished and there are no masses of material of different nature. The aggregations are generally of circular form, the diameter is 1 μ. Sometimes a one layer limiting membrane is evident. Their opacity varies depending on the degree of compactness of the ferritin molecules.

The ... Dr. W. B... ... Dr. A. M. J... of the Electron Microscopy Section ... for ... Cancer ... Service ... gratefully acknowledged.



Fig 1 Erythroblast from normal subject. In the cytoplasm numerous dispersed ferritin molecules are present. The invaginations of the cell membrane and the vacuoles with ferritin (arrows) demonstrate an active rophcocytosis. The mitochondria are of normal aspect. Electron micrograph, 16 000 ×

(fig 7) c) *Masses of mixed materials* They are composed partly of ferritin molecules, still distinguishable, partly from small compact masses of high electronic opacity and partly from various substances of minor opacity These masses have irregular forms. Their dimensions vary between 0.3 and 3 μ They are often limited by an one-layer membrane (fig 7)

The aggregates of the ferritin molecules and the masses of mixed materials correspond to the Perls-positive granules of 'hemosiderin This substance has, in fact, a complex and variable chemical composition (13–16)

In the reticular cells the iron accumulations are often seen inside the phagosomes. The mitochondria do not show any accumulation of iron and their morphology is almost unaltered.



Fig 2. Erythroblast from patient with refractory sideroblastic anaemia (the following figures refer to the same patient Around the nucleus (N) there are some mitochondria (m) which contain important accumulations of iron. The vesicles of the endoplasmic reticulum are dilated. The dispersed ferritin molecules are less numerous in this cell than in the near reticular cell (RC The mitochondria of the latter cell do not contain iron accumulations. Electron micrograph, 14 000

2. *In the cytoplasm of the erythroblasts the dispersed molecules* of ferritin are sometimes highly increased in number In other erythrocytes their number is few (fig. 2,3 4) These differences have no evident links with other ultrastructural patterns. Aggregates of ferritin molecules are present, but in less quantity than in the reticular cells. We have not seen any masses of mixed materials.

The characteristic pictures of rhopheocytosis are very frequent (fig 4) The ferritin molecules here are arranged along the



Fig 3. Erythroblast in more advanced stage of maturation. The nucleoplasm (N) is less opaque. The mitochondria are overcharged with compact and highly opaque deposits of iron, which hide the morphology of the organelles. Endoplasmic reticulum is dilated. Electron micrograph, 15.000 ×

wall. In our preparations rhopheocytosis does not appear particularly frequent in the zones facing towards the near reticular cells.

In all the erythroblasts the mitochondria are overcharged with iron in a clearly elective manner (fig 2 3 4) The iron here is arranged in compact accumulations that are between the cristae (fig 5) These accumulations are composed of a fine powdery substance. It was impossible for us, as also for other authors (6 17) to recognise the ferritin molecules in this material, for which Bessis proposed the term "iron micelles" In many erythroblasts this pathological concentration of iron affects all the chondrioma. For the perinuclear disposition of the mitochondria these accumulations resemble a crown around the nucleus. They clearly correspond to the pathological images of ringed sideroblasts. The alterations of the mitochondria are already present in the more immature cells. Alterations of this type are occasionally observed in the pro-erythro-



Fig 4 Portion of the cytoplasm of erythroblast. The lesion affects all the mitochondria. The iron aggregates are evident. Ferritin molecules are present in the vacuoles and in the invaginations of the cell membrane (arrows) Electron micrograph, 36 000 ×

blasts (18) in which an accumulation of iron is also noted using histoautoradiographic techniques (19 20)

In our preparations we have never seen the zones of amorphous substance nor the dense spherules observed in the cytoplasm of thalassaemic erythroblasts (21)

The alterations provoked in the erythroblasts by the metabolic defects vary from cell to cell. In one part of the erythroblasts, in different phases of maturation, the general morphology of the cells is not modified Sometimes the endoplasmic reticulum appears di



Fig. 5. The iron accumulations in mitochondria are less consistent here. The deposits are between the cristae. Electron micrograph, 60 000

lated. On the contrary in other erythroblasts some regressive processes which reach as far as the cellular lysis are seen. The alterations develop until the whole cytoplasm is transformed into an amorphous material, while the nucleoplasm becomes homogeneous and disintegrates. The reticular cells show a marked overcharge of iron in the cytoplasmic zones in contact with the dissolving erythroblasts (fig. 7). A systematic examination of our preparations indicates that these processes are of a higher frequency than normal. This suggests an increased intramarrow lysis of the erythroblasts.



Fig. [illegible]. Two reticulocytes (R), one erythrocyte (E) and one erythroblast (M). The images of rhopheocytosis with ferritin (arrows) are numerous in the reticulocytes. Electron micrograph. [illegible]

3. In [illegible] the remaining mitochondria show iron accumulations similar to those described in the erythroblasts. Numerous dispersed ferritin molecules are often observed. Rhopheocytosis with ferritin is present in a part of the reticulocytes, and in some cells the process appears intense (Fig. 6). The identification of reticulocytes was well established, as stated above, comparing each ultrathin section with the successive semithin section.

Hepatic tissue. Under the light microscope, the general structure of the tissue appears to be conserved. A moderate hyperplasia of the connective tissue is present in the portal tracts. The parenchymal cells are overcharged with a yellow granular Perls-positive pigment. This pigment is particularly abundant around the hepatic vein, around the portal tracts and in the connective tissue. The Kupffer cells also are overcharged with iron. The tissue does not show diffused or significant aspects of secondary [illegible].

Under the electron microscope, the following alterations are seen (Fig. 8–1[illegible]).

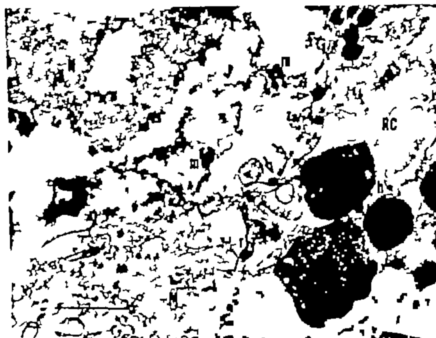


Fig 7 Contact-zone between an erythroblast in advanced lysis and reticular cell (RC). The nucleoplasm (N) of the erythroblast is disorganized and homogeneous. I the cytoplasm the fragments of mitochondria overcharged with iron (m) are still recognizable. This zone of the cytoplasm of the reticular cell is particularly rich in ferritin molecule aggregates (f) and in mixed masses (h). Along the cell membrane numerous small masses of highly opaque and complex material are evident (arrows) Ferritin molecules and small masses are contained in rhopheocytosis images ( ) All these aspects are absent in the contact-zone between the erythroblast and a near myeloblast (M) Electron micrograph, × 18 000

1 In the *parenchymal cells* numerous dispersed ferritin molecules are observed They are sometimes evident in the Disse space and in the lumen of the bile canaliculus. The mitochondria of the liver cells never show accumulations of iron The general aspect of the cytoplasm is characterised by the presence of numerous ferritin aggregates and masses of mixed materials. A one layer limiting membrane is sometimes easily visible The ergastoplasm is well developed and rich in ribosomes. The distribution of glycogen does not show significant modifications. At the level of the cell membrane the microvilli of the Disse space and of the bile canaliculus have normal aspects.

2. The *Kupffer cells* are frequently thickened and protrude into the sinusoidal lumen. In their cytoplasm the iron appears as ferritin

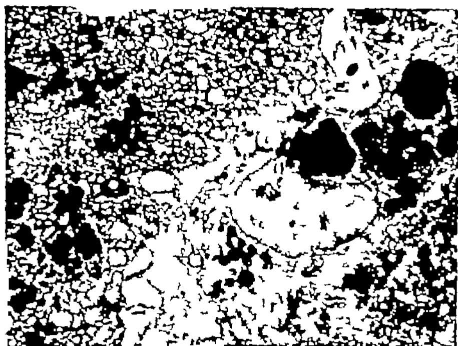


Fig. 8. In hepatic tissue the iron overcharge is evident in the parenchymal cells as ell as in the Kupffer cells (K.) The latter are thickened and often occupy a large part of the sinusoidal lumen. I their cytoplasm large part of iron is contained in the phagosomes (F) which are increased in number and size. In the parenchymal cells the glycogen is bundant. This substance is here opaque and appears as diffused granules. The numerous iron accumulations are highly opaque. The mitochondria of neither type of cell contain iron accumulations. I the Disse space reticulin fibers (r) are evident. Electron micrograph, 4 000 x

dispersed molecules as ferritin aggregates and as masses of mixed materials. In these cells the accumulations of iron are more important than in parenchymal cells. The accumulations are often enclosed in the phagosomes. The mitochondria do not show any iron accumulations.

Discussion

More of our ultrastructural observations on idiopathic sideroachrestic anaemia are not in contrast with those reported for other six cases (1–6). The sideroblastosis is characterized by an elective iron overcharge in the mitochondria. A large part of the iron accumulated in the erythroblasts is found inside these organules, between the cristae. The iron here has a finer structure than the ferri-

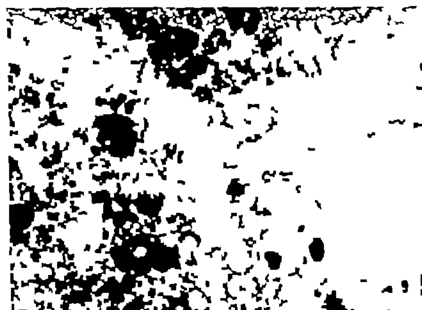


Fig. 9. I. Liver cells, ferritin aggregates (f) and more opaque masses, in which the ferritin is irregularly mixed with heterogeneous substances (h) are seen. The difference of the size between ferritin molecules and glycogen (g) is evident. The mitochondria have normal aspects. Electron micrograph, 5.000 x

tin molecule. Some important reactions of porphyrin metabolism occur at the level of the erythroblastic mitochondria (22–25). In normal conditions these iron accumulations are usually absent because of the existing metabolic equilibrium. In refractory sideroblastic anaemia a mitochondrial enzyme defect upsets this equilibrium. Under the electron microscope we observe the iron unutilized and accumulated between the cristae. This is probably seen near the places in which the blocked enzymatic reaction occurs.

At present it is difficult to establish a direct and certain relationship between the enzyme defects so far identified and the accumulation of iron in mitochondria. The accumulations are present also in thalassaemias (21) and in the erythroblasts of the normal guinea-pig (26). The accumulations are, however, absent in lead poisoning (27, 28) and in infectious anaemias (17). They are only occasionally observed in normal human erythroblasts and in idiopathic haemochromatosis (2, 17). A systematic study of the ultrastructural, as well as biochemical aspects could probably establish more precise links between the morphological and func

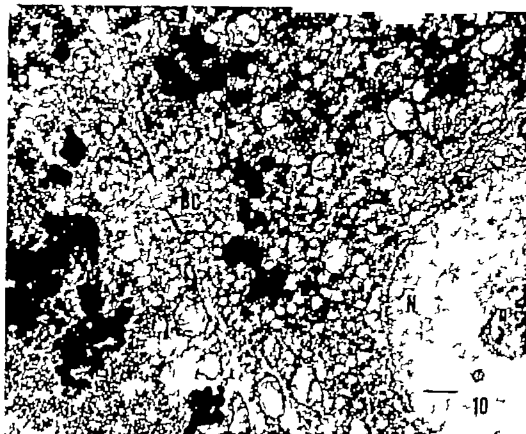


Fig 10 The iron accumulations are often arranged around the bile canaliculus (BC) The morphology of this appears normal. The cytoplasm is rich in glycogen. On the right the ucleus (N) and the nucleolus ( ) of parenchymal cell are visible. Electron micrograph, 7 000

tional modifications, contributing to a more exact knowledge of hypochromic hypersideraemic anaemias.

The erythroblast, which have a high speed metabolism and an active proliferation, must necessarily suffer from a diffused and consistent alteration of its mitochondria. An exaggerated ineffective erythropoiesis, caused by an intramarrow lysis of an aliquot of affected cells, is suggested by the observed increase of the stercobilinogen elimination (7 29) Lysis of erythroblasts is frequently seen in our preparations The iron of lying cells is taken by the near reticular cells. These aspects suggest an important biological cycle of the iron inside the bone marrow

The exchanges of iron at the level of the cell membrane of the erythroblasts appear to us as rhopheocytosis (fig 1 4 6) which is not a process specific for the iron. In iron-deficiency anaemias rhopheocytosis without ferritin is seen (30) Other authors have discussed the true direction of rhopheocytosis. The problem is whether this process always indicates an absorption of the substance, or whether these aspects can also indicate elimination of ferritin from the erythroblasts (17 31) In our preparations we have not observed a greater number of rhopheocytosis profils in the parts of the cell membrane of erythroblast which are in contact with the reticular cells. Besides, rhopheocytosis is intense not only in the sideroblasts but also in a part of the medullar siderocytes. These latter cells are already rich in haemoglobin and a great number of dispersed molecules of ferritine is evident in their cytoplasm. At this stage of maturation the presence of an intense rhopheocytosis seems to suggest two-way exchanges of the iron rather than a paradoxical avidity of the cell. Nevertheless, at present it is difficult to give a certain ruling as the the rhopheocytosis processes, outside of particular experimental conditions.

The aspects of the hepatic tissue in the siderosis secondary to the refractory sideroblastic anaemia are similar to those observed in the idiopathic haemochromatosis (2, 10 32) In the parenchymal and Kupffer cells the iron assumes three forms of aggregation the dispersed ferritin molecules, the aggregates of ferritin and the masses of iron mixed with other substances. Many aspects suggest the possibility that these different forms could be successive phases of iron aggregation in the cytoplasm, as suggested by other authors (6 33 34) The iron accumulations are often limited by an one-layer membrane. In the Kupffer cells, as in reticular cells of the bone marrow the phagosomes are increased in number and size, and contain a good part of the iron. Once activated, these cells have a remarkable capacity for accumulating and storing various substances. The phagosomes isolate the accumulated substances inside themselves. Similar patterns have been observed by us in the human liver tissue in other pathological conditions (35)

Summary

The bone marrow and liver tissue of patient suffering from non hereditary idiopathic form of refractory sideroblastic anaemia were examined under optical and electron microscopes. Important iron accumulations in the mitochondria of erythro-

blasts are observed. Rhopheocytosis with ferritin ppears intense in erythroblasts. The process is present in part of the medullar reticulocytes also. Lysis of erythroblasts is *frequently seen. The iron of lysing cells is taken by the reticular cells.* The reticular cells of bone marrow the Kupffer cells and the liver cells are overcharged with iron. I these cells iron accumulations in mitochondria are never seen

Résumé

On a examiné microscope optique et microscope électronique la moelle et le tissu hépatique d'un cas d'anémie réfractaire sidéroblastique idiopathique non-héréditaire. Des remarquables accumulations de fer ont été bservées dans les mitochondries des érythroblastes. La rophéocytose avec ferritine est bien evidente dans les érythroblastes et dans une partie des réticulocytes médullaires. La lyse des érythroblastes est fréquente. Le fer des ellules en lysis est capté par les cellules réticulaires. Les cellules réticulaires de la moelle osseuse, les cellules de Kupffer et les cellules hépatiques sont surchargées de fer On 'y pas observé des accumulations dans les mitochondries.

Zusammenfassung

Knochenmark und Lebergewebe eines Patienten mit einer nicht hereditären, idiopathischen Form einer refraktären sideroblastischen Anämie wurden licht und *elektronenmikroskopisch untersucht. In den Mitochondrien der Erythroblasten fanden* sich bedeutende Eisenansammlungen. Diese Zellen zeigten eine intensiv Rhopheocytose fur Ferritin. Der Prozeß kommt uch in einem Teil der Mark Retikulocyten vor Eine Lyse von Erythroblasten tritt haufig in Erscheinung, wobei das Eisen der aufgelosten Zellen von Reticulumzellen aufgenommen wird. Die Reticulumzellen des Knochenmarkes, die Kupffer-Zellen und die Leberzellen sind mit Eisen überladen. I diesen Zellen finden sich jedoch keine Eisenansammlungen in Mitochondrien.

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Studies of Basophils

The Effects of Exogenous Heparin Upon the Number and Morphology of Basophils*

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The substance which causes the metachromasia of tissue mast cell granules was identified by Holmgren and Wilander in 1937 as Heparin (1) In human blood basophils heparin like substances have also been found though there is still some controversy about their true identity (2 3 4) In recent years, several authors have studied the effect of exogenous heparin upon the number of circulating basophils. Angeli et al (5) reported an increase in healthy male subjects within two hours after a single injection of heparin Braunsteiner and Thumb (6) saw under similar experimental conditions no significant change in the mean number of basophils but with repeated injections of a long acting heparin over several days, a significant reduction occurred from the third day on. In the rabbit the results of a single injection of heparin have been more uniform Jucker (7) Boseila (8) and Piette and Piette (9) observed a drop in numbers during the first few hours after an intravenous injection of heparin No information regarding basophil morphology after heparin treatment has been available until now

In this study the in vivo and in vitro effects of exogenous heparin upon the numbers and morphology of human and rabbit basophils are presented.

Materials and Methods

In vivo studies For the study of human basophils blood from 16 young healthy adults, 12 male and 4 female was used. Several blood examinations were made before and after the period of experimentation. Seven adult male and 7 adult female rabbits were used in the animal studies. They had been under close observation including numerous blood examinations for an average period of two years. The heparin employed was sterile commercial solution of sodium heparin with an average potency of 130

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units USP per mg. Further dilutions when necessary were made with physiological saline or Ringer' solution.

Six male subjects received between 10,000 and 12,500 units and 8 rabbits 500 units of heparin in single intravenous injection. The injections were made in the morning hours. Blood was drawn immediately before and thirty minutes, 4 and 24 to 48 hours afterwards.

In vitro studies Blood samples were incubated with heparin in test tubes at concentration of 10 units per ml blood or in heparinized capillary tubes. Incubation time was 2 hours at 37 C. Samples were removed after 30 minutes, one hour and tw hours.

White cells and basophils were counted in more than 100 samples and basophil morphology studied in smears made from the same samples. Aliquots of blood from these samples were made incoagulable with sodium citrate and incubated simultaneously as controls.

The clotting time of venous blood was determined by the LEE nd WHITE method and of capillary blood by McGOWANS technique. Recalcification time was measured by the HALSE method.

Elimination of heparin and uro-heparin was estimated by comparing the metachromasia produced by toluidine blue in the urine of 4 male subjects after the heparin injection with standard series of heparin-toluidine blue solutions made with the subjects own urine oided immediately before heparin was given. Basophils were counted in Speirs-Levy chambers using IHAGAKI' fluid for human basophils and solution of toluidine blue in acetic acid for rabbit basophils (11) Differential counts of basophils were made in smears stained with WRIGHT's, PAPPENHEIM s, or UNDRITZ toluidine blue stain.

Results

In Vivo Studies

Humans Plasma recalcification times were determined four times in each of four male subjects and compared with the numbers of basophils, before administration of heparin No correlation was found in the samples studied

Whole blood coagulation times of blood from six male subjects were longest 30 minutes after the i.v heparin injection and varied between 23 minutes and 2 hours 44 minutes. Urine samples from four subjects showed that maximal excretion of heparin and uroheparin took place during the first 30 minutes only traces of metachromasia remained after 4 hours and none could be detected after 24 hours. Basophil numbers dropped after 30 minutes in three rose in two subjects and remained the same in one person After four hours they were lower in two higher in three and not altered in one. Only slight alterations of the total white cell counts were observed after the injections. No significant correlation at the 0 05 level was found between white cell and basophil counts before and after the injection

Rabbits Eight adult rabbits received 500 units of heparin After 30 minutes the number of basophils was lower in four higher

in three animals and not counted in one rabbit. After four hours basophil numbers were lower in all eight rabbits. The reductions were significant in each animal. 24 hours after the injection lower basophil numbers were found in two in the other six a significant rise above pre injection levels had occurred Total white cell counts varied considerably during the experiments. No significant correlation existed between their numbers and those of the basophils before and after the injection.

In a second series, six rabbits received 500 units of heparin and ten minutes later 5mg protamine sulfate both by the intravenous route. After 30 minutes a rise in basophil numbers was noticed in four animals, in two the number had dropped After four hours three animals had higher and three lower basophil counts.

Table I

Changes in number size and position of the granules and position of the nucleus of human and rabbit basophils after in vivo and in vitro treatment with heparin.

Granules (Distribution in percent)

Human Basophils	Number			Size			Position		
Before	numerous	medium	few	coarse	med.	small	«P»	I	U
Heparin	16	70	14	17	70	13	18	28	54
After heparin	17	67	16	46	43	11	17	36	47
After incub. with heparin	6	64	30	33	59	8	*23*	43	34
Rabbit Basophils									
Before heparin	18	75	7	14	71	15	*7*	22	71
After heparin	16	78	6	26	67	7	*9*	38	53
After incub. w heparin After 20 min	14	63	23	25	60	15	*33*	60	7
After 60 min							*55*	40	5

position of the granules was determined as «P» (peripheral) «I» (intermediate) and «U» (uniform)

the figures in italics designate cells with their nucleus in an excentric position.

In Vitro Experiments

All blood samples remained unclotted during the time of observation.

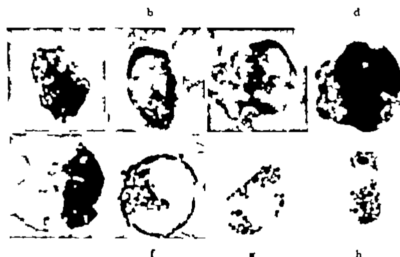


Fig 1 Morphology of Basophils after treatment with heparin.) and *b*) Human basophils after incubation with heparin; *d*) and) rabbit basophils after incubation with heparin;) human basophil after i. inj. of heparin *f*) rabbit basophil after i. inj. of heparin *g*) and *h*) rat mast cells after s.c. inj. of heparin.) *f*) (1500)) and *b*) reduced for comparison with *g*) and *h*) which were draw after microfotos (×1050) from HILL AND PORTER (31)

Human blood After one hour incubation with 10 units heparin per ml blood there was no significant change in the number of white cells and basophils in the samples of capillary and venous blood taken from eleven subjects After two hours incubation the total number of white cells was reduced in some samples by 2000 per mm³ No significant change had occurred in the numbers of basophils. After incubating blood from one male subject with heparin for 24 hours the following changes were observed Before incubation 6,950 wbc, 34 basophils per mm³ blood after 4 hrs incub 5 150 wbc, 16 basophils per mm³ after 24 hours incub 5,250 wbc, 16 basophils per mm³

Rabbit blood After incubating several samples of venous blood from eight male and female rabbits with 10 u heparin per ml blood no significant change in the white blood cell count was found after two hours. Basophil counts had remained the same in 20 samples, in two a drop of 34 and 24 / respectively had occurred In several samples the concentration of heparin was increased to 250 units per ml without a change in white cell and basophil numbers after 20 minutes incubation.

Basophil Morphology

The examination of 450 human and 557 rabbit basophils in the stained smear for their morphology after in vivo and in vitro treatment with heparin revealed the following cytological changes.

Cell size and shape Alterations of cell diameters were variable and within narrow limits. Some cells appeared larger others shrunk. The majority of the diameters were between 9 and 15 microns A tendency for polymorphism was observed in a number of cells. Unusually oblong or ameboid shapes with pseudopod like extensions of the cytoplasm were noticed occasionally (figs. 1a, b c)

Basophilic granules The changes produced by heparin in per centage distributions of the basophils classified by the various characteristics of their granules are summarized in table I

The distributions described in table I were compared using the KOLMOGOROV-SMIRNOV two sample test with a 5 % level of significance.

Number of granules There was no significant difference between the distributions of number of granules before and after the i.v injection of heparin for either human or rabbit basophils. However after incubation with heparin both in human and rabbit basophils, distributions of the number of granules differed markedly from those before heparin and after the intravenous injection of it. After incubation the proportion of human and rabbit basophils with few granules had increased.

Size of granules Both the injection of heparin and the incubation of blood with it produced in human and rabbit basophils a significant change in the distribution of all three sizes of granules. Cells with coarse granules became relatively more frequent while the proportions of those with medium and small sized granules decreased. In many instances these coarse granules appeared swollen, their borders ill defined and frequently clumped together Often dark metachromatically stained vacuoles appeared to have replaced them (fig 1d)

Position of granules After the i.v injection of heparin a moderate but not significant increase in the proportion of human basophils with granules in the intermediate position took place, while the relative number of peripheral forms remained practically unchanged. In rabbit basophils a significant shift to «P» and «I» forms was observed.

Position of nucleus Incubation of blood with heparin caused in some human and rabbit basophils a displacement of the nucleus to an excentric position which in its turn affected the position of the granules. These cells resembled juvenile rabbit basophils or human basophilic myelocytes (11) (fig 1f) However concomitant alterations in cell size and shape and in the cytoplasm allowed the distinction from normal immature cells The proportion of cells with a lateral nucleus increased with time of exposure to heparin in the incubation experiments after 20 minutes every third rabbit basophil showed such alterations, after one hour every second cell was affected. On the other hand the varying concentrations of heparin between 2 and 275 units per ml blood did not appear to influence the degree of the changes.

Vacuolization and metachromasia Besides the darkly stained vacuoles which replaced the granules in some cells after treatment with heparin other vacuoles appeared which seemed empty These were usually larger and more numerous than the clear vacuoles often seen in normal basophils (fig 1c) In some cells a definite reduction in metachromatic staining or a complete loss of metachromasia was observed (fig 1e) This was most noticeable in cells with few and small granules but there were also basophils in which small pink granules lay next to large darkly stained granules. Occasionally after prolonged incubation the nucleus took up the metachromatic stain and appeared darker than the granules (fig 1e)

The above described morphological changes did not seem to affect more than half of the basophils in the majority of the preparations two out of three basophils appeared unaltered No similar changes were seen in the control samples incubated with sodium citrate.

Heparin produced in vivo and in vitro changes in other white cells besides the basophils. Only the lymphocytes appeared entirely unaffected

Discussion

Basophil numbers The effect of single injections of heparin upon the numbers of human and rabbit basophils were similar to those reported by the majority of previous observers. In man, in agreement with the findings by Braunsteiner and Thumb (6) no consistent changes in the number of circulating basophils could be observed during the first four hours after an intravenous injection.

In vivo treatment of rabbits with heparin brought confirmation of the findings reported in the literature (7 8 9) Rabbits responded with a marked reduction of basophil numbers four hours after a *single intravenous injection.* A *neutralizing dose of protamine* sulfate given 10 minutes after heparin failed in half of these cases to prevent a drop in basophil numbers. Heparin induced reductions of basophil numbers rarely lasted beyond 24 hours.

In the in vitro experiments the numbers of basophils appeared unaffected by the exposure to various concentrations of heparin during the first 2 hours of incubation. BOSEILA AND ASBOE HANSEN (12) found after 20 minutes incubation with 250 units heparin per ml rabbit blood a decrease in white and basophil numbers. This they attributed to the strong affinity of heparin for the toluidine blue in their diluting fluid which prevented staining of the basophils. Differences in the degree of acidity between their diluting fluid and ours may explain the divergent results. The PIETTES (13) have shown that rabbit basophils stain better at a lower pH. Our diluting fluid had a pH of 2.5 BOSEILA s modification of the MOORE AND JAMES formula one of 7.5. It may therefore, be that due to the favorable acid milieu in our diluting fluid all the basophils were stained before the excess of heparin bound most of the toluidine blue.

In approaching the question of the significance of the quantitative and qualitative changes produced by heparin in basophils one must consider their possible role within and outside of the circulatory system. The heparin contents of basophils would seem to point to a connection with hemostatic processes. There are no records which correlate an increase in basophil numbers with a lengthening of blood clotting time. Length of plasma recalcification times in the male subjects of this study did not show any correlation with the numbers of basophils found in the same samples of blood.

The basophils of two young healthy males counted repeatedly during the first 10 minutes after an intravenous injection of heparin did *not* vary significantly while at the same time there was a 100 to 400 / increase in the clotting time of the blood. On the other hand the reduction in basophil numbers persisted in the rabbits beyond the peak of the heparin induced hypocoagulability of the blood. At the present stage of investigation the evidence which connects variations in basophil numbers during their passage

through the blood stream with the anticoagulant activity of heparin is controversial.

There are data which correlate basophils with lipid metabolism. In diseases with a disturbed fat metabolism such as diabetes, hypothyroidism and nephrosis their number has been found to be increased But also here conditions are at this moment far from clear BRAUNSTEINER et al (6) did not find a correlation between basophil numbers and cholesterol levels nor was there any numerical change during alimentary lipemia (6 14)

ASBOE HANSEN (15) and RILEY (16) and their schools consider the main field of heparin activity to be in the connective tissue where it takes part in the laying down of the ground substance through the mast cells. Investigations by REBUCK et al. (17) into the role of basophils in inflammatory processes have demonstrated simultaneous states of heightened activity of both blood basophils and mast cells. These observations prompt one to raise again the question of the possible participation of blood basophils in tissue processes in which histamine and mucopolysaccharides of the heparin type are involved.

Basophil morphology The alterations in morphology of human and rabbit basophils under heparin treatment resembled in some instances those described in tissue mast cells of the rat and mouse after heparin (fig 1a, b g, h) However any interpretation of such similarities should take into consideration the possibility of spontaneous cytological variations species differences in basophils, and morphological and cytochemical differences between mast cells and basophils.

Spontaneous morphological alterations have been observed in both blood basophils and tissue mast cells. ZITCER et al (18) saw in cultures of human mast cells a cyclic production and loss of granules. Seasonal changes in the number and position of granules in blood basophils and tissue mast cells have been seen in mammals during hibernation (19)

Changes in the position of basophil granules in accordance with cell age have been observed and described by the writer (11 20) Degranulation with and without cell disintegration has been produced in basophils of several species by treating them with histamine liberators or heparin antagonists (21 22 23 24) These processes at times were accompanied by violent shock like reactions

due to either the compounds themselves or the release of material such as histamine from the cells.

Commercial heparin though not identical with the endogenous heparin of man and rabbit is even when used in excessive quantities free of the toxic effects of its antagonists and the histamine releasers, and capable of preventing the lethal shocks these compounds produce in some laboratory animals (25 26 27) One must, therefore, assume that the action of heparin is of a different nature. The literature contains several descriptions of the penetration of heparin into body cells without causing them to disintegrate (28, 29 30 31 32) Jorpes (33) has commented upon the relative ease with which cells accommodate a substance of such high molecular weight as heparin and attributes this to the low osmotic pressure heparin possesses in spite of its high negative electric charge.

Our own observations on the preservation of basophil numbers after incubating blood for two hours with enough heparin to prevent its coagulation for 24 hours and longer speak against a disruption of basophils by heparin though an application of morphological changes produced in vitro to in vivo processes must be made with reserve. In addition, the basophil being a rather fragile cell easily suffers mechanical injuries which may result in extrusion of cytoplasm and scattering of granules. These accidental alterations are *often hard to distinguish from the effects of the specific agent* employed. This does not detract from the value of the study of in vitro produced morphological alterations provided manipulations of the basophils are kept at a minimum. Degranulation and loss of metachromasia by basophils in vivo are not necessarily followed by cell death and disintegration as will be demonstrated elsewhere (34)

The changes in cell shape and the position of the nucleus have their parallel in recent observations by Bell and Jeon (35) who describe the effects of heparin upon cell locomotion and pseudopod formation of ameba proteus. They attribute this effect to a depolarization of the anterior portion of the ameba and assume that heparin might produce similar effects upon leucocytes in their natural environment. During locomotion basophils carry the nucleus in the forward part (36) It, therefore appears possible that the appearence of basophils with an excentric nucleus after incubation with heparin is the result of such a depolarization process. It would seem reasonable to contend that basophils normally take part in the

absorption of heparin, its transportation to the tissues and its deposition there. The changes exogenous heparin produced in basophils may be considered as the model of such an in vivo process in which basophils participate in the economy of heparin.

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Summary

A single intravenous injection of heparin caused in rabbits reduction in the number of circulating basophils, in healthy human males the results were inconsistent. Incubating human and rabbit blood with heparin produced no numerical reductions of basophils. Morphological changes in human and rabbit basophils after incubation with heparin were similar but not identical with cytological alterations after in vivo treatment with heparin. The changes concerned numbers, size and position of the granules, position of the nucleus and metachromatic staining. The possible mechanism of the alterations and their significance with regard to basophil and heparin economy are discussed.

Résumé

Une injection intraveineuse unique d'héparine produit une diminution du nombre des basophiles du sang périphérique chez le lapin. Les résultats obtenus chez des mâles adultes ne sont pas uniformes. L'incubation de sang humain ou du lapin avec de l'héparine ne produit pas de diminution du nombre des basophiles. Les modifications morphologiques des basophiles de l'homme et du lapin ressemblent, sans être identiques, aux modifications cytologiques après injection d'héparine. Ces modifications concernent le nombre, la taille et la localisation des granulations, la localisation du noyau et la métachromasie. Les auteurs discutent les mécanismes possibles des modifications et leur signification en relation avec l'économie des basophiles et de l'héparine.

Zusammenfassung

Einmalige intravenöse Injektion von Heparin führte bei Kaninchen zu einer Verminderung der zirkulierenden Basophilen. Bei gesunden männlichen Versuchspersonen waren die Ergebnisse uneinheitlich. Die Inkubation von menschlichem und Kaninchen-Blut mit Heparin ergab keine Abnahme der Basophilenzahl. Die morphologischen Veränderungen der Basophilen von Mensch und Kaninchen nach Inkubation mit Heparin waren ähnlich aber nicht identisch mit den zytologischen Veränderungen nach Zufuhr von Heparin in vivo. Die Veränderungen betrafen Zahl, Größe und Lage der Granula, Lage des Kerns und Metachromasie. Die möglichen Mechanismen der Veränderungen und ihre Bedeutung im Hinblick auf den Umsatz von Basophilen und Herapin werden erörtert.

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Haemophilia Due to Combined Deficiency of AHG, PTC and PTA Factors*

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Since 1803 when Otto (1) first described haemophilia, until a few years ago the disease was thought to occur exclusively in males, while transmitted by female carriers. In 1947 Pavlovsky (2) observed, that blood or plasma of some haemophiliacs had the property of correcting another haemophiliac's clotting anomaly. The question of the participation of more than one factor in the pathogenesis of haemophilia has been thus for the first time opened to discussion.

In 1952, Aggeler et al. (3) Biggs et al. (4) as well as Schulman and Smith (5) independently described haemophilia due to another antihaemophilic factor which Aggeler called plasma thromboplastin component (PTC) and Biggs Christmas factor. In 1953 Rosenthal et al. (6) drew attention to the existence of another factor they named plasma thromboplastin antecedent (PTA) the deficiency of which also caused haemophilia.

There are still investigators who disagree with those attributing haemophilia to the deficiency of a definite factor and attribute the disorder in the presence of inhibitors (7) in the patient's blood.

Since 1953 on the grounds of pathogenesis, three forms of the disease have been distinguished, haemophilia A, B, and C, due to the deficiency of AHG, PTC and PTA factors respectively whereas only a few cases of haemorrhagic disorder due to a combined deficiency of two factors have been described (8–16).

This paper refers to a patient with haemorrhagic disorder due to the deficiency of all three factors, that is AHG, PTC, and PTA.

* Aided by the Department of Clinical Research, Lederle Laboratories, New York, USA.

Case Report

K. N aged 28, claims left knee joint haemarthrosis first ppearing t the age of 7 following an injury. Since then, frequent relapses have been appearing at the same joint, during the subsequent tw years. At the age of ten, his right knee joint and afterwards, all bigger ones were affected at intervals. For some years extensive haematomata and ecchymoses have set in.

Routine blood examinations have shown nothing of particular interest. The number of platelets (280.000 per mm³) as well as their shape the fragility of the capillaries (tourniquet test) bleeding time, clot retraction and plasma prothrombine time were all within normal limits. The whole blood clotting time repeatedly examined was found to be greatly prolonged (1 to 3 hours).

Table I

Type of mixture	Clotting time (Lee-White) minutes (normal: up to 20 min)	Serum prothrombine time, 60 min after clot formation, seconds (normal above 20 s)	Test for circulating anticoagulant
Normal blood	14	68	
Patient' blood (without any addition)	>150	7.5	negative
Patient's blood after addition of			
NaCl 0.9%	>160	9	
normal plasma	8	72	
normal $BaSO_4$-plasma	20	27.5	
normal serum	23	23	
normal $BaSO_4$-serum	31	19	

0.1 ml reagent to 0.9 ml tested blood.

Special Blood Examinations

The results of clotting studies are summarized in table I. Taking into consideration that the correction of the blood clotting time and prothrombine consumption could be obtained both by serum and by barium sulphate plasma the above results would lead to the diagnosis of haemophilia C, due to the deficiency of the PTA factor But the following points are not compatible with this type of haemophilia a) The patient's clinical picture, who as mentioned, suffered from heavy haemophilic syndrom, while it is known that haemophilia C runs course with slight symptoms of haemorrhagic diathesis or more frequently the haemorrhagic diathesis is manifested after certain surgical intervention (17 18). b) The extremely prolonged clotting time (17 18)) Upon addition of serum or barium sulphate plasma, neither clotting time nor prothrombine consumption exhibited any satisfactory correction. Therefore, we have repeatedly (ten times in all) examined the patient' clotting anomaly various intervals both t the phases of remissions and exacerbations of the haemorrhagic disorder W have also examined the patient' blood by crossing it at various proportions with the blood of known haemophiliacs suffering from haemophilia A, B and C, respectively W have also repeatedly examined the thromboplastin generation test and studied the possibility of the existence of circulating anticoagulant in the patient blood. Moreover, we have sent twice patient's plasma for examination to Dr R. Rosenthal, New York, and have twice received from him sample of PTA deficient plasma for crossmatching studies.

Table II

Effect of normal, AHG deficiency PTC deficiency and PTA deficiency plasma and serum as well as BaSO plasma and serum on clotting and serum prothrombin times of our patient.

Type of substance	Clotting time (Lee-White) minutes (normal up to 20 min)	Serum prothrombine time, 60 min after clot formation, seconds (normal above 20)
Patient's blood (without any addition)	>180	8.5
Patient's blood after addition of		
NaCl 0.9%	>180	9
normal plasma	11	65
normal $BaSO_4$-plasma	20	20
normal serum	21.5	18
normal $BaSO_4$-serum	29.5	19
haemophiliac's A (AHG deficiency) plasma	21.5	21
haemophiliac A $BaSO_4$-plasma	48	16.5
haemophiliac's A serum	19	20.2
haemophiliac' A $BaSO_4$-serum	55	14 4
haemophiliac B (PTC deficiency) plasma	23	16.2
haemophiliac B $BaSO_4$-plasma	21	15.4
haemophiliac's B serum	65	12.2
haemophiliac's B $BaSO_4$-serum	68	10
haemophiliac' C (PTA deficiency) plasma	16	35
haemophiliac's C $BaSO_4$-plasma	29.5	19
haemophiliac' C serum	60	12
haemophiliac' C $BaSO_4$-serum	>120	6.5
Dr Rosenthal's (PTA deficiency) plasma	18	45

0.1 ml reagent to 0.9 ml tested blood.

The results of crossmatching studies and of thromboplastin generation are reported in tables II to V and figures 1 to 4 respectively

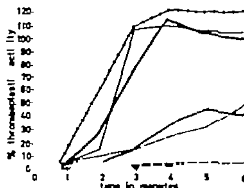
A circulating anticoagulant in our patient's blood has been excluded (table VI)

Family Studies

The father of the patient had 4 sisters and 4 brothers. The patient's father died in 1949 from cancer of the liver When alive he did not show any haemorrhagic signs. One of the sisters shows haemorrhagic tendency while one of the brothers, we examined (table VII, E. N.) manifests posttraumatic haemorrhagic syndrom.

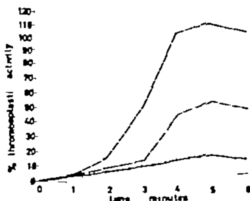
A sister of the patient died 25 years ago at the age of 2, from haemorrhagic syndrom. Patient' mother and one of her sisters, aged over 60, had history of menorrhagia. The patient has a 32-years-old brother who has not presented so far any haemorrhagic manifestations.

The blood laboratory findings of the patient siblings and relatives, whom we have been able to examine, are given in table VII and fig 5 to 8. With the exception of case 4 the other three cases show slight delay of the clotting time and a defective prothrombine consumption as well. Both return back to normal after addition of



Normal platelets + normal $BaSO_4$-plasma + normal serum
Patient platelets + patients $BaSO_4$-plasma + patient's serum
Normal platelets + patient $BaSO_4$-plasma + patient's serum
Patient's platelets + normal $BaSO_4$-plasma + patient' serum
Patient's platelets + patient $BaSO_4$-plasma + normal serum
Patient's platelets + normal $BaSO_4$-plasma + normal serum
Patient' platelets + normal plasma + patient's serum

Fig. 1 Thromboplastin generation test.



Patient' platelets + patient $BaSO_4$-plasma + patient' serum
Patient' platelets + patient $BaSO_4$-plasma + normal serum
Patient' platelets + normal $BaSO_4$-plasma + patient's serum
Patient's platelets + our case's $BaSO_4$-plasma + patient' serum
Patient' platelets + our case's $BaSO_4$-plasma (during the haemorrhagic crisis of June 1957) + patient's serum

Fig. 2. Thromboplastin generation test of patient with AHG deficiency

Table III

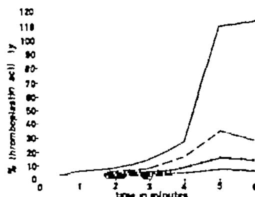
Effect of our patient plasma and serum on clotting and serum prothrombin times of persons with severe AHG, PTC and PTA deficiency

Type of mixtures	Clotting time (Lee-White), minutes (normal up to 20 min)	Serum prothrombin time, 60 min after clot formation, seconds (normal above 20)
I. Haemophilia A (AHG deficiency)		
Haemophiliac blood (without any addition)	160	7.5
1 ml. haemophiliac blood after addition of		
0.1 ml normal $BaSO_4$-plasma	10	34
0.1 ml propositus $BaSO_4$-plasma	62	9.2
0.3 ml propositus $BaSO_4$-plasma	31.5	13.4
0.5 ml propositus $BaSO_4$-plasma	20	19.6
1.0 ml propositus $BaSO_4$-plasma	13	31
II. Haemophilia B (PTC deficiency)		
Haemophiliac's blood (without any addition)	120	10
1 ml. haemophiliac' blood after addition of		
0.1 ml normal serum	9	62
0.1 ml propositus serum	80	9.8
0.5 ml propositus serum	26.5	18.2
1.0 ml propositus serum	14.5	26.4
III. Haemophilia C (PTA deficiency)		
Haemophiliac blood (without any addition)	31	14.2
1 ml. haemophiliac blood after addition of		
0.1 ml normal $BaSO_4$-plasma	8	48.2
0.1 ml propositus $BaSO_4$-plasma	29.5	15.2
0.5 ml propositus $BaSO_4$-plasma	18.5	17.4
1.0 ml propositus $BaSO_4$-plasma	16	22.4
0.1 ml normal serum	11	50
0.1 ml propositus serum	29.5	13.4
0.5 ml propositus serum	20.5	17.2
1.0 ml propositus serum	15	24.2

normal individual's barium sulphate plasma or serum. Thromboplastin generation test in all four cases is defective because in no case the quantity of thromboplastin produced exceeds 50% of the normal, after 4 minutes.

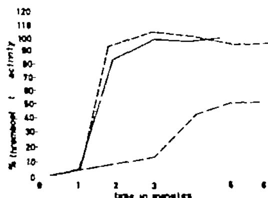
Discussion

If frequency of haemophilia A is 1 10 000, that of B (PTC deficiency Christmas disease) 1 100 000 and that of haemophilia C (PTA deficiency) still lower then the combined deficiency of the two factors shall be extremely rare, whereas that of all three factors practically impossible. In view of these facts we have repeatedly controlled our patient s haemorrhagic disorder both during



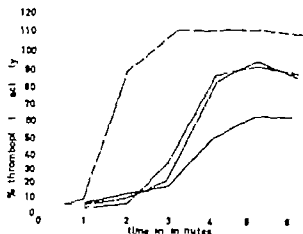
—— Patient platelets + patient's $BaSO_4$-plasma + patient serum
—— Patient platelets + patient $BaSO_4$-plasma + normal serum
— — — Patient's platelets + normal $BaSO_4$-plasma + patient' serum
— — — Patient's platelets + patient's $BaSO_4$-plasma + our case' serum
—·—·— Patient' platelets + patient $BaSO_4$-plasma + our case's serum (during the haemorrhagic crisis of January 1956)

Fig 3. Thromboplastin generation test of patient with PTC deficiency



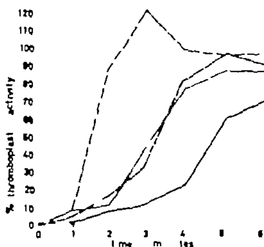
—— Patient' platelets + patient' $BaSO_4$-plasma + patient serum
—— Patient' platelets + patient's $BaSO_4$-plasma + normal serum
— — — Patient' platelets + normal $BaSO_4$-plasma + patient' serum
— — — Patient' platelets + our case's BaSO -plasma + patient serum

Fig 4 Thromboplastin generation test of patient with PTA deficiency



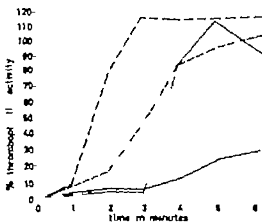
——— Patient platelets + patient's BaSO$_4$-plasma + patient serum
——— Patient's platelets + patient BaSO$_4$-plasma + normal serum
——— Patient' platelets + normal BaSO$_4$-plasma + patient's serum
— — — Patient's platelets + normal BaSO$_4$-plasma + normal serum

Fig. 5. Thromboplastin generation test propositus F N (patient' mother)



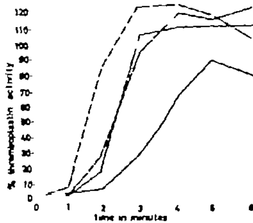
——— Patient' platelets + patient BaSO$_4$-plasma + patient's serum
——— Patient platelets + patient' BaSO$_4$-plasma + normal serum
- - - - Patient' platelets + normal BaSO$_4$-plasma + patient serum
— — — Patient' platelets + normal BaSO$_4$-plasma + normal serum

Fig. 6. Thromboplastin generation test propositus Ch. N. (patient's brother).



——— Patient' platelets + patient' $BaSO_4$-plasma + patient serum
——— Patient's platelets + patient $BaSO_4$-plasma + normal serum
– – – – Patient's platelets + normal $BaSO_4$-plasma + patient's serum
– – – Patient's platelets + normal $BaSO_4$-plasma + normal serum

Fig. 7 Thromboplastin generation test propositus E. N. (father's brother)



——— Patient platelets + patient $BaSO_4$-plasma + patient' serum
——— Patient platelets + patient BaSO -plasma + normal serum
——— Patient' platelets + normal $BaSO_4$-plasma + patient serum
——— Patient's platelets + normal BaSO -plasma + normal serum

Fig. 8 Thromboplastin generation test propositus E. D. (mother' sister).

Table IV

Cross-matching studies on our patient blood during haemorrhagic crisis.

Type of mixture	Clotting time (Lee-White), minutes (normal, up to 20 min)	Serum prothrombine time, 60 min after clot formation, seconds (normal above 20)
Haemorrhagic crisis (April 1957)		
Patient blood (without any addition)	>240	8.2
1 ml. patient's blood after addition of		
0.1 ml normal plasma	9.5	41.4
0.1 ml normal $BaSO_4$-plasma	20.5	21.2
0.1 ml normal serum	>180	9.4
0.1 ml normal $BaSO_4$-serum	>180	7.6
Haemophiliac's A (AHG deficiency) blood	140	8.8
1 ml. haemophiliac's blood after addition of		
0.1 ml normal $BaSO_4$-plasma	8	41.2
0.1 ml normal serum	150	10.2
0.1 ml propositus $BaSO_4$-plasma	155	9.6
0.5 ml propositus $BaSO_4$-plasma	160	8.6
1.0 ml propositus $BaSO_4$-plasma	150	9.2
Haemophiliac' B (PTC deficiency) blood	120	11.2
1 ml. haemophiliac's blood after addition of		
0.1 ml normal BaSO -plasma	150	11.8
0.1 ml normal serum	7.5	39.2
0.1 ml propositus serum	62	12.4
0.5 ml propositus serum	21	18.2
1.0 ml propositus serum	15	20.8

remission and haemorrhagic crises. Our patient s haemophilia is not due to the deficiency of the AHG or PTC factor as the correction of his blood clotting time and prothrombine consumption did not occur by a mere addition of normal barium sulphate plasma or serum respectively Haemophilia C (PTA deficiency) should be excluded among other reasons (clinically severe haemorrhagic syndrom, protracted clotting time) from the very fact that plasma of a C haemophiliac (PTA deficiency) restored back to normal our patient s blood coagulation. This has been verified repeatedly twice by C haemophiliac s plasma (PTA deficient plasma) sent to us by Dr Rosenthal and twice by our patient s own plasma, which was forwarded to Dr Rosenthal, New York, for examination

The following findings are against the combined total deficiency of AHG and PTC factors a) Addition of our patient s barium sulphate plasma or serum at a relatively high dose (1 ml) corrects

Table V

Cross-matching studies on our patient blood during haemorrhagic crisis.

Type of mixture	Clotting time (Lee-White) minutes (normal up to 10 min)	Serum prothrombine time, 60 min after clot formation, seconds (normal above 20 s)
Haemorrhagic crisis (January 1959)		
Patient's blood (without any addition)	>180	10.2
Patient's blood (1 ml.) after addition of		
0.1 ml normal plasma	6.5	31.4
0.1 ml normal $BaSO_4$-plasma	>180	12.2
0.1 ml normal serum	20	19.4
0.1 ml normal $BaSO_4$-serum	>180	9.2
0.1 ml Dr. ROSENTHAL's (PTA deficiency) plasm	19	21.5
Haemophiliac's A (AHG deficiency) blood	145	6.4
1 ml. haemophiliac's blood after addition of		
0.1 ml normal $BaSO_4$-plasma	9.5	29.6
0.1 ml normal serum	1.0	7.2
0.1 ml propositus $BaSO_4$-plasma	110	7.6
0.5 ml propositus $BaSO_4$-plasma	21.5	17.4
1.0 ml propositus $BaSO_4$-plasma	14	20.8
Haemophiliac' B (PTC deficiency) blood	85	10
1 ml. haemophiliac's blood after addition of		
0.1 ml normal $BaSO_4$-plasma	90	10.2
0.1 ml normal serum	6.5	31.6
0.1 ml propositus serum	88	10.8
0.5 ml propositus serum	85	11.8
1.0 ml propositus serum	75	11.2

Table VI

Test for circulating anticoagulant (calcium clotting time)

	Proportions of mixture				
Patient' plasma, ml	0	0.1	0.5	0.9	1
Normal plasma, ml	1	0.9	0.5	0.1	0
Clotting time, seconds	112	114	122	136	>600

both clotting time and prothrombine consumption of A and B haemophiliacs. b) In the thromboplastin generation test of patients suffering from haemophilia A or B, the replacement of barium sulphate plasma or the serum, by the aforesaid reagents respectively taken from our patient, causes a partial thromboplastin formation.

For similar reasons, the haemorrhagic disorder of our patient is not due to any combined total deficiency of AHG and PTA factors, neither to that of PTC and PTA ones. The combined partial deficiency of AHG and PTC factors is not compatible with our

Table VII
Results of clotting studies in our patient family

Type of mixture	Clotting time (Lee-White), minutes (normal up to 10 min)	Serum prothrombine time, 60 min after clot formation, seconds (normal above 20 s)
1 Propositus F V (mother)		
propositus blood (without any addition)	22	16.8
propositus blood after addition of:		
normal $BaSO_4$-plasma	13	28.2
normal serum	11.5	30.2
2 Propositus Ch. N (brother)		
propositus blood (without any addition)	23.5	14.6
propositus blood after addition of		
normal $BaSO_4$-plasma	14	24.4
normal serum	12.5	26
3 Propositus E. N (father' brother)		
propositus blood (without any addition)	24	13.2
propositus blood after addition of		
normal $BaSO_4$-plasma	11	30.6
normal serum	12.5	32.4
4 Propositus E. D (mother's sister)		
propositus blood (without any addition)	14	30.4
propositus blood after addition of		
normal $BaSO_4$-plasma	10	31.2
normal serum	8	30.2

patient as PTA factor was also missing (see findings on table III) Our patient s plasma, twice controlled by Dr ROSENTHAL, New York, the first time did cause a partial, the second time no correction at all of the clotting time and of the prothrombin consumption of known C haemophiliacs. Moreover a C haemophiliac thromboplastin formation became completed if his serum or barium sulphate plasma were replaced by corresponding ones of a healthy individual and defective if by those of our case. In a previous report (18) it has been proven that PTA factor is essential for the production of complete blood thromboplastin. For quite similar reasons our patient s haemorrhagic disorder is not due to a combined partial AHG and PTA or PTC and PTA factors deficiency

The presence of a circulating anticoagulant in the patient s blood should also be excluded as shown after thourough laboratory investigation. If anything like that happened, the concentration of the anticoagulant whould be greater in the patient's plasma than in his serum, since the various anticoagulative substances become

partially inactivated during the progress of the coagulation process. Such an anticoagulant we were not able to demonstrate in our patient's plasma.

Our case of haemophilia could not be explained otherwise, unless we accept that AHG PTC and PTA factors, essential for the production of complete thromboplastin during the blood coagulation process, are contained in our patient's blood in reduced concentrations. The addition of only one of the missing factors to the patient's blood causes no correction at all in the clotting time and /or prothrombine consumption. The addition of two factors affords an incomplete correction to the clotting time and prothrombine consumption.

Addition of normal plasma, i. e. of all three factors (AHG PTC and PTA) causes complete correction of the patient's blood coagulation. The addition of the two factors AHG and PTC (C haemophiliac's plasma—PTA deficiency) also involves correction of both clotting time and prothrombine consumption. This has been verified also in our C haemophiliacs and in the case of Dr ROSENTHAL as well. It is possible that the PTA factor concentration in the patient's blood during the time of examination was lower than normally but it was sufficient enough as to make normal the clotting time and the prothrombine consumption.

The concentration of the AHG PTC and PTA factors in the patient's blood was not constant on the examinations made at various intervals and has presented instead considerable fluctuations. During two severe haemorrhagic crises the patient's haematological behaviour was in the first, as if he suffered from haemophilia A, while in the second he behaved like a haemophiliac B.

Up to the present there have been 9 well defined cases of haemophilia reported due to a combined deficiency of two factors. But as the incidence of haemophilia A is 1 10 000 that of B 1 100 000 and that of C 1.500 000 the possibility of coexistence of the genes responsible for haemophilia in one and the same individual or in individuals of one and the same family ought to be as rare as practically never to occur In order to explain the existence of haemophilia due to the combined deficiency of two factors we should probably admit that a mild deficiency of either factor is much more common than it is commonly accepted but such a deficiency is not sufficient enough to cause any clinical manifestations unless by chance they occur in combination on the same in-

dividual. Besides the question is whether the factors AHG PTC and PTA are physiologically linked in a way which is not apparent yet. In such a case haemophilia A, B and C from the pathogenetical point of view would be nothing but variants of one and the same basic disorder of clotting process.

Summary

A case of a male is described, presenting from his childhood clinical symptoms of classic haemophilia. Special examinations revealed that the patient' haemorrhagic diathesis was due to a combined deficiency of the AHG PTC and PTA factors. Some of his family members exhibited mild disturbance of blood coagulation, obviously due to the partial deficiency of one or more factors. Nine cases of haemophilia due to deficiency of more than one factor reported so far in the literature are reviewed and the possible explanation of the relatively fragment appearence of such combination from the pathogenetical point of view is discussed.

Résumé

Les auteurs rapportent le cas d'un homme qui montrait depuis son enfance les symptomes cliniques d une hémophilie classique. L étude de la coagulation montrait un manque combiné des facteurs AHG PTC et PTA. Quelques membres de la famille souffrent de troubles légers de la coagulation, dûs au manque partiel d'un ou de plusieurs de ces facteurs. 9 cas d'hémophilie causés par le manque de plus d'un seul facteur sont rapportés de la littérature. Puis une explication possible pour l'existence relativement fréquente d'un défaut combiné est discuté du point de vue de la pathogénèse.

Zusammenfassung

Es wird über einen männlichen Patienten berichtet, der seit seiner Kindheit die klinischen Symptome einer klassischen Hämophilie aufwies. Die gerinnungsphysiologischen U tersuchungen ergaben einen kombinierten Defekt der Faktoren AHG, PTC und PTA. Einige Familienmitglieder zeigten leichte Gerinnungsstörungen, die durch partiellen Mangel eines oder mehrerer Faktoren bedingt waren. Aus der Literatur werden 9 Fälle von Hämophilie zusammengestellt, die durch das Fehlen von mehr als einem Faktor bedingt waren. Die mögliche Erklärung für das relativ häufige Vorkommen einer solchen Kombination wird vom Standpunkt der Pathogenese aus diskutiert.

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Quantitative Cell Counts of the Bone Marrow and Blood and their Secular Variations in the Normal Adult Rat

By E. V. Hulse

The advent of simple methods for assessing the cellularity of bone marrow (7 11 12, 31 35) allows detailed quantitative assessments of haemopoiesis in experimental animals. During a quantitative study of the changes in haemopoiesis after irradiation (17 18, 19 20 21 22) a total of 81 normal unirradiated rats were examined by a method based on that of Yoffey (36) As comparatively little has been published on values obtained using quantitative methods and as the present series is much larger than those previously published, an analysis of the data from the normal rats has been made. In addition certain aspects of the techniques for absolute counts of the bone marrow were subjected to statistical examination

Materials and Methods

Male rats of an inbred albino strain were used throughout. I the initial series of experiments 40 normal animals were examined during period of 15 months and in the second series 41 normal animals were examined during 22 months, with 5 month gap between the two series. All the animals were adult when examined, those in series I being from 3½ to 7¼ months old (average age 5 months) and those in series II from 3 to 5 months (average age 4 months). Each animal came from different litter

After the animals were anaesthetized with sodium pentobarbitone, the chest was opened and sample of blood taken from the right entricle. Each animal was then exsanguinated by cutting one of the larger branches of the aortic arch. Both femurs were removed and split longitudinally and the marrow removed from the shaft, care being taken not to include spicules of bone. The marrow from one bone was used for cell counts and that of the other for smears and specific gravity determinations. After the blood and marrow samples had been taken each animal was autopsied and no naked-eye pathological changes were found in the unirradiated animals.

Blood Counts. Red cell counts were performed using haemocytometer and Hayem' solution. Tw pipettes were taken from each sample two rulings were counted from each pipette and the counts veraged. Haemoglobin was measured using an M.R.C. grey wedge haemoglobinometer (24) and an a erage of 3 readings were taken. Reticulocyte counts were performed only on blood from animals in the second series the preparations were made by placing drop of blood on slide on which an 0.5 % alcoholic solution of brilliant cresyl blue had previously dried. The number of reticulocytes

amongst one thousand red cells was counted. Packed cell volume measurements were made on the last 26 animals of series I and the whole of series II.

Turk's solution was used for the total white cell count and the counting procedure was the same as for the red cells. A differential count was done on 200 cells from Jenner-Giemsa stained film. Platelet counts, using the method of Brecher and Cronkite (4) were performed only on animals from series II.

Marrow-Cell Counts: In principle, sample of marrow is dispersed in a suitable *fluid by vigorous shaking. In the original method (36) the dispersing fluid was serum.* The following fluid, however was found to give better dispersion and was less opaque when staining fluid was added prior to counting:

dipropylene glycol	25 ml
20% (W/V) solution of dihydric sodium citrate	15 ml
distilled water	to 100 ml

Exactly 1 ml of this fluid was put in a small stoppered bottle (capacity about 2 ml) which was then weighed. Marrow was then placed in the bottle and shaken vigorously in mechanical shaker for about 5 minutes and then weighed again. The marrow sample usually weighed 20–30 mg and in order to calculate its volume the specific gravity of the marrow was determined by releasing small pieces of marrow from glass rod beneath the surface of range of copper sulphate solutions of known specific gravity (16)

The specific gravity of normal marrow from the femur humerus and tibia ranged from 1.056 to 1.070.

A staining fluid was made from dispersing fluid by adding 1% (V/V) of 40% formaldehyde and convenient amount of gentian violet. 0.2 ml of the marrow suspension was diluted with 0.4 ml of staining fluid and the number of nucleated cells and red blood cells were then counted in an improved Neubauer chamber A chamber with metallized surface made the counting easier The numbers of cells per cmm of marrow were calculated from counts on 6–10 rulings.

When films are made from solid marrow i. e. marrow removed from split bone rather than as an aspirate, the Romanowsky type of stain tends to give "methylene blue-eosin effect. The true Romanowsky effect can, however be retained if the solid *marrow is diluted with serum (9), bovine albumin (2) or plasma substitutes (13) In* the present instance smears were made by chopping up small portions of marrow in drop of pooled rat serum at one end of glass slide and spreading the resulting suspension in the usual manner for blood films. Good dispersion and satisfactory Jenner-Giemsa staining was thus obtained. Differential counts were performed on one thousand nucleated cells. The absolute number per cmm was then calculated for each type of marrow cell.

Test of Methods for Quantitative Marrow Counts: Three problems posed by the methods were investigated. These were 1) whether exsanguinating the animal t the beginning of the experiment alters the red cell content of the marrow 2) whether the cellularity of the right femur is the same as that of the left and 3) whether femoral marrow is, in the rat, truly representative of marrow elsewhere.

The effect of exsanguination can be tested in two ways, either by removing one femur and then exsanguinating the animal before removing the femur of the opposite side or by examining femurs from two animals of the same litter one of which had been exsanguinated and one not. The former comparison is more direct but entails the possibility that surgical shock from the removal of one femur even with full anaesthesia and surgical haemostasis, might so alter the circulation as to prevent full exsanguination. Therefore both methods were used, four comparisons being made in each case. The cellularity of the right and left femurs were also compared in four rats.

The method of marrow counting can be used only in situations where there is sufficient quantity of marrow free of bone. In the rat only the femur tibia and humerus

Table I

Effect of exsanguination on the number of red cells in femoral marrow (counts given as millions per mm³ of marrow).

	Exsanguinated	Not exsanguinated
Left femur removed before exsanguination	0.29	0.67
	0.61	0.56
	0.57	0.93
	0.95	0.57
Litter mates, one exsanguinated, one not exsanguinated	0.70	0.74
	1 18	0.95
	1 05	1 13
	1 19	1 07

Table II

Comparison of the number of nucleated cells and red blood cells in the femur humerus tibia in normal and irradiated rats (count in millions per cmm of marrow).

	Nucleated Cells			Red Blood Cells		
	Femur	Humerus	Tibia	Femur	Humerus	Tibia
Normal rats	2.22	2.58	2.29	0.863	0.244	0.750
	2.50	2.59	2.71	1.09	0.440	1.26
	3.20	3.37	3.01	0.855	0.650	0.720
	2.67	2.59	2.76	0.770	0.173	0.540
Irradiated rats	1.04	1.20	1.32	0.760	0.343	0.860
(24 hours after 200)	1.81	2.23	1.88	1.61	0.860	1 13
	1.53	1.83	1.68	1.34	0.895	1.24

are suitable and four rats were used for comparisons between the three bones. The same techniques were used except that the smaller amount of marrow in the tibia and humerus necessitated the original marrow suspensions being diluted 1 1 with staining fluid instead of 1:2 as used for the femur The same comparisons were also made in three rats given 200 of X-rays 24 hours before killing.

Results

Test of Quantitative Marrow Techniques The effects of exsanguination of the red cell content of the femoral marrow are given in table I The data were subjected to analyses of variance and t tests but no statistical differences were revealed. Thus there was no evidence that exsanguination reduced the amount of peripheral blood in the bone marrow

Comparisons between the numbers of nucleated cells in right and left femurs gave the following results right femurs 2.81 2.86, 2.45 and 2.92 million cells per cmm, left femurs 2.94 2 78, 2 62 and 2.64 million cells per cmm. Analysis of variance and t tests on these data showed no significant difference between the two sides. The animals used were those in which one femur was removed

before and one after exsanguination but the exsanguination should not have made any appreciable alteration in the numbers of nucleated cells as there are about three hundred times as many nucleated cells per unit volume in the marrow as in the blood.

Comparisons between the femur humerus and tibia are given in table II Statistical tests (analysis of variance and t' tests) on the data for the nucleated cell count in normal animals did not reveal any significant difference between the marrow of the three bones. The same tests showed that after irradiation also the marrow of the three bones did not differ in their nucleated counts. The number of red cells per cmm of marrow from the femur and tibia were statistically similar in both normal and irradiated rats. However the concentration of red cells in the marrow of the humerus was significantly less than that for the femur both in normal rats and in irradiated rats. There appears, therefore, to be a reasonable degree of uniformity in the nucleated cell content of the marrow from different long bones but differences may exist in the non-nucleated red cell content of marrow from different bones.

Table III

Cellularity of normal rat marrow: numbers of cells in millions per mm³ (standard errors in parentheses)

	Series I		Series II	
Number of rats	40		41	
Total nucleated cells	2.35	(0.04)	2.59	(0.04)
Non-nucleated red cells	0.752	(0.060)	0.642	(0.037)
Total marrow cells	3.10	(0.08)	3.23	(0.06)

This figure is significantly greater ($P < 0.001$) than that for series I.

Quantitative Data. Total marrow counts are given in table III absolute values for the various types of marrow cell in table IV and absolute values for blood in table V Percentages based on the mean counts are also given, where appropriate. The total counts and absolute values for the two series were compared statistically using the t test, and values for P are given when these were 0 05 or below It is often presumed that differences are statistically significant when P = 0 05 but in the present instance when over 20 different comparisons are being made such a difference could occur by chance. For this investigation, therefore, attention was paid only to differences for which P was 0 02 or less.

A search was made for possible causes for the differences between the two series. It is well recognised that lymphoid tissue de-

Table IV

Cellular analysis of normal rat bone marrow: absolute numbers in thousands per cmm, standard errors in parentheses, values of 'P' for the absolute numbers are given when 0.05 or less.

	Absolute numbers Series I		Series II		P	Percentages Series I	Series II
Total Erythropoetic Cells	676	(20)	748	(22)	0.02	28.7	28.9
Late Normoblasts	379	(15)	428	(15)	0.02	16.1	16.5
Intermediate Normoblasts	247	(10)	248	(10)		10.5	9 6
Early Normoblasts	40.9	(3.2)	51.8	(2.8)	0.02	1 7	2.0
Pronormoblasts	9.54	(0.75)	20.7	(1.5)	0.001	0.4	0.8
Total Myelopoietic Cells	917	(27)	869	(20)		39.0	33.6
Segmented Neutrophils	423	(13)	415	(14)		18.0	16.0
Juvenile Neutrophils	84.2	(5.0)	118	(6)	0.001	3.6	4.6
Metamyelocytes	70.1	(4.3)	54.9	(2.7)	0.01	3.0	2.1
Myelocytes	192	(9)	137	(6)	0.001	8.2	5.3
Promyelocytes	35.5	(3.2)	30.9	(2.4)		1.5	1.2
Eosinophils	103	(5)	105	(5)		4.4	4.0
Basophils	9 4	(1.2)	10.1	(1.2)		0.4	0.4
Blast Cells	58.9	(3.0)	50.3	(2.6)	0.05	2.5	1.9
Lymphocytes	424	(17)	627	(25)	0.001	18.0	24 2
Monocytes	47 7	(4.4)	42.0	(2.9)		2.0	1.6
Megakaryocytes	9.80	(1.5)	9.48	(1 4)		0.4	0 4
Mast Cells	18.5	(2.5)	26.3	(3.5)		0.8	1.0
Plasma Cells	24.3	(1 7)	15.9	(1.5)	0.001	1.0	0 6
Reticulum Cells	79.3	(5.8)	74.5	(6.3)		3.4	2.9
'Smear' Cells	76.4	(7 1)	113	(6)		3.3	4.3
Myeloid-Erythroid Ratio			–			1.36	1 16

Table V

Absolute values and percentages for the blood of normal adult rats, standard errors in parentheses. Unless otherwise stated the absolute numbers denote cells per cmm. Values of 'P' for comparisons of the absolute numbers given when 0.05 or less.

	Absolute numbers Series I	Series II	P	Percentages Series I	Series II
Total White Cells	6040 (250)	7150 (240)	0.02		
Segmented Neutroph.	1040 (90)	879 (115)		17.3	12.4
Juvenile Neutrophils	151 (17)	172 (21)		2.5	2.5
Eosinophils	104 (12)	78 (11)		1 7	1 1
Lymphocytes	4450 (260)	5790 (190)	0.001	73.9	81.6
Monocytes	275 (22)	117 (14)	0.001	4.6	2.3
Platelets		838 (29) $\times 10^3$			
Erythrocytes	8.2 (0 1) $\times 10^6$	8.3 (0.1) $\times 10^6$			
Reticulocytes				–	2.3 (0.3)
Haemoglobin g/100 ml	15.4 (0.2)	15.5 (0.2)			
Packed Cell Volume %				49 (1)	52 (1)
M.C.H.C., %				31.8 (0.8)	29 6 (0.4)
M.C.H., $\mu\mu g$	19 1 (0.4)	18.7 (0.3)			
M.C.V. μ^3	60.4 (1.5)	63.3 (1 1)			

Haemoglobin was measured as percentage and in order to express it in g/100 ml it has been assumed that the conversion factor for rat haemoglobin is the same as that for human haemoglobin.

Table VI

Mean counts of lymphocytes in blood and femoral bone marrow at various ages, standard errors in parentheses.

Age in months	Number of Animals		Blood Lymphocytes per mm³		Marrow Lymphocytes, thousands per mm³	
	Series I	Series II	Series I	Series II	Series I	Series II
3	3	19	7050 (90)	6040 (300)	575 (56)	683 (30)
4	17	21	4550 (420)	5530 (270)	433 (21)	599 (24)
5	9	1	4470 (400)	6620	434 (42)	722
6	7	–	3630 (440)	–	373 (39)	–
7	4		3480 (910)	–	345 (35)	

creases in amount with age. The present data were, therefore, examined to see whether the differences in lymphocyte count could be explained by differences in age animals from series I being, on average, one month older than those from series II The distribution of animals between the various age groups is far from equal but some indication of lymphocyte changes with age may be obtained from table VI. In series I there was a progressive decrease in both blood and marrow lymphocytes with increasing age and in series II, which virtually consisted of animals aged 3 and 4 months, the same trend is apparent. However when the 4 month old animals (i. e. the largest age group in both series) are compared the lymphocyte counts for series II are greater than those for series I and it must be concluded that the differences in lymphocyte counts are not wholly due to age.

Other cell series did not reveal any changes associated with age. The differences must, therefore, be regarded as a secular variation. The same strain of inbred rats were used throughout and the only major alteration in the strain which occurred during the experimental period was the process of making it free of ectoparasites. This was done by thorough treatment of a litter of weanling rats with di (parachlorophenyl)-methylcarbinol after which the litter was used as the breeding nucleus for the ectoparasite free animals. The change was accomplished during the period covered by series I and the thirtieth animal of series I and all subsequent animals were ectoparasite-free. Cell counts from the eleven ectoparasite-free animals of series I were examined separately and compared with the series II results. Table VII lists those cell types in which it was found that the counts from the ectoparasite-free animals of series I were not significantly different from those of series II animals but were significantly different from the counts of the 29 other rats of

Table VII

Variations in the bone marrow and blood of series I which occurred when the strain of rats was made free of ectoparasites, standard errors in parentheses. The cell counts in the two columns are significantly different from each other but the counts from the ectoparasite-free animals were not significantly different from the same counts in series II (see tables IV and V).

	Ectoparasites present	Ectoparasites absent	P
Number of rats	29	11	–
Total erythropoietic cells (in thousands per mm³)	650 (21)	746 (42)	0.05
Late normoblasts (in thousands per mm³)	352 (14)	488 (29)	0.002
Metamyelocytes (in thousands per mm³)	79.8 (4 4)	44.6 (4.9)	<0.001
Myelocytes (in thousands per mm³)	208 (10)	148 (14)	0.002
Total white cells of the blood (cells per mm³)	5550 (250)	7580 (400)	0.001
Blood lymphocytes (cells per mm³)	3890 (3)	5930 (4)	<0.001

series I. This suggests that the process of making the strain free of ectoparasites in some way led to minor changes in haemopoesis.

Morphological Observations The haemopoietic cells of the rat were found to differ from those of many other mammals in two ways.

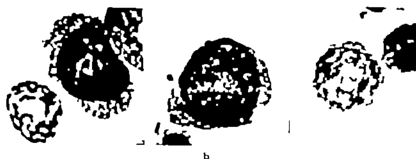
1) The cytoplasm of the erythroid series in the rat retains an element of basophilia much later than the corresponding cells in man. Thus it was inappropriate to classify normoblasts as basophilic, polychromatic and orthochromatic and the alternative classification of early intermediate and late normoblasts (23 34) was used. The most mature cell of the series, the late normoblast, has cytoplasm which varies from slightly basophilic to polychromatic. This tendency to retain the basophilic elements longer was seen amongst the non-nucleated red cells, the numbers of polychromatic cells being much greater in the rat than in man, a point which was noted by Creskoff et al (6)

2) In the rat the nuclei of some of the immature cells of the granulocyte series have a hole in the centre. Such nuclei have been called doughnut or ring' forms and it has been stated that the doughnut form is intermediate between the myelocyte and the metamyelocyte (33) In the present series of observations it was noted that the hole in the nucleus is not confined to one particular stage of maturation. Two kinds of gr ,te cel ade by

b

d f

Fig 1 Maturation of neutrophilic cells of the granulocyte series with 'doughnut' or 'ring' nuclei. The size of the hole in the nucleus increases as the cell matures (a–f). Jenner-Giemsa. ×1800.



b

Fig 2a, b Cells of the eosinophil series showing holes in their nuclei similar to those of the neutrophil series. Jenner-Giemsa 1800

side, one which develops a hole in the centre of the nucleus and one which does not. The hole first appears as a very small opening in the nuclei of some promyelocytes, i. e cells with azurophilic granules but few if any specific granules. As the cells mature the hole becomes larger until, as a late metamyelocyte, the cell has a narrow ring-like nucleus (fig 1) Thus, with practice, it would be possible to estimate the maturity of this type of cell by inspecting the size

of the central hole of the nucleus using a purely nuclear stain such as FEULGEN's.

No ring forms were seen amongst the few basophils which are present in rat marrow but the eosinophil series show the same type of holing of the nucleus as the neutrophil series (fig. 2).

Discussion

The lack of any statistical difference between the marrow of the right and left femurs of the rat and the similar finding of HARRIS et al. (15) in respect to the right and left humerus of the guinea pig suggest that, in the normal animal, active marrow from one bone may be presumed to be the same as that from the same bone on the contralateral side.

The aim of the exsanguination of the animal in the original technique (36) was to cut down contamination of the marrow with peripheral blood. Exsanguination will remove peripheral blood from any soft tissue organ which is able to contract (e. g. spleen and liver) and so compensate for the loss of the volume of blood it contained. The bone marrow, however, is an organ set within a hard unyielding framework and simple exsanguination would, on theoretical grounds, appear unlikely to effect its blood content. It is not, therefore, surprising that exsanguination did not alter the number of red blood cells in the marrow (table I). Consequently, although it was regularly used this part of the technique is unnecessary.

The nucleated cell count was similar in the femur, tibia and humerus (table II) and it may therefore be concluded that in this respect the femoral marrow is representative of marrow in the long bones. However, the red cell counts in the humerus were consistently lower than those of the femur and tibia. It seems very unlikely that the difference is due to differences in red cell production as the total nucleated counts were the same. It is more likely to be due to differences in circulation or in the size and number of blood vessels and sinusoids.

Secular Variations in Blood and Marrow Counts. Statistical variations in cell counts between the two series are noted in tables III–V. Lymphoid tissue is known to vary in quantity with age but the difference in mean age between the two series was only one month and the present data (table VI) showed that such an age difference

was insufficient to account for the differences between the two groups. No positive correlation with age could be found in any other cell type showing secular variations and in some instances the variations were not associated with any known change in the strain of rats used. However changes in the numbers of those types of cell listed in table VII were associated with the establishing of a colony free from ectoparasites. As this entailed restarting the colony from a very small nucleus of animals, it is possible that there was accidental selection in favour of animals with a higher number of late normoblasts and a smaller number of metamyelocytes and myelocytes in the marrow and a higher number of blood lymphocytes.

Since the change to ectoparasite free animals it has become apparent that the strain now has a higher incidence and a six month earlier onset of the rat kidney disease termed nephrosis by SAXTON AND KIMBALL (32). It is possible that there was also accidental selection in favour of animals which developed nephrosis earlier at the time of establishing the ectoparasite-free colony. This can be taken as possible selection in favour of more than one change of characteristic. It is also possible to envisage an interrelation between the kidney condition and the normoblast changes. None of the animals used in the present investigation had any naked-eye evidence of kidney disease but it is not outside the realm of possibility that some change in erythropoietin levels could occur before there was anatomical evidence of kidney damage.

A further possibility is that the absence of ectoparasites in itself could result in the changes noted in table VII. When series I was started the strain was infested with the rat louse. The infestation was never heavy in young adults but was present throughout the colony. If the changes listed in table VII were due to the presence of a small number of lice it would be necessary to presume that there was a demand for red cells in the peripheral blood which resulted in a reduced pool of late normoblasts in the marrow. Shortly before series I was commenced the strain was found to be free of Bartonella infection and it seems unlikely that the bites of a few lice should cause a change in the number of late normoblasts. Also it would be necessary to make the presence of the lice responsible for the increase in myelocytes and metamyelocytes and this again seems very unlikely particularly as there was no neutrophilia to denote an infection in series I. Thus accidental selection appears to be the most likely reason for the variations. If this is so the data a e of

interest in demonstrating how genetic variations can cause minor alterations in the constituents of a non pathological tissue

These secular variations emphasise the need for frequent observations on control animals during haematological experiments. Where possible it is highly desirable that the control animals should be litter mates of the experimental animals.

Marrow Cellularity Little work has been reported on quantitative assessments of the cellularity of rat bone marrow and few of the published series concern more than ten adult animals Methods using camera lucida tracings were used by KINDRED (25) and ROOFE et al (30) Techniques similar to the one used in the present investigation were used by FRUHMAN AND GORDON (11) MANTZ (26) OSOGOE AND AWAYA (27) BURKE AND HARRIS (5) RIMAN et al (29) BIERRING (3) and RAMSELL AND YOFFEY (28) Total nucleated counts (means and standard errors) in agreement with those given in table III were obtained by most of the above investigations, viz. $2\ 43 \pm 0\ 08 \times 10^4$ (3) $2\ 4 \pm 0\ 11 \times 10^4$ (11) $2\ 25 \pm 0\ 10 \times 10^4$ (28) $2\ 05 \times 10^4$ (8) $1\ 81 \pm 0\ 05 \times 10^4$ (27) and $1\ 79 \pm 0.81 \times 10^4$ (26) cells per cmm and $2\ 46 \pm 0\ 19 \times 10^4$ (5) and $2\ 05 \pm 0\ 07 \times 10^4$ (29) cells per mg of marrow A much higher value of $3\ 98 \times 10^4$ cells per cmm was obtained by ROOFE et al (30)

Marrow Differential Counts RAMSELL AND YOFFEY (28) list differential counts from the literature The present values (table IV) fall well within the ranges given In view of the secular changes noted in the present investigation it is not surprising that there should be a fairly wide range of values noted in the literature.

Blood Counts The red cell count reticulocyte percentage, haemoglobin and packed cell volume (table V) were all within the range of those reported by previous workers (1 6 8 10 14 30) The white cell count in the present animals agrees with that given by DONALDSON (8) and falls within the range quoted by CRESKOFF et al. (6) It is, however lower than those of some other workers whose counts range from 14 750–20 100 per mm^3 (10 15 30)

Differential counts of rat blood in the literature give varying proportions of the different types of white blood cell, some of which are close to the values given in table V The secular changes in the present series suggest that variations in differential counts can be expected not only in different strains but also in the same strain at different times.

Acknowledgments: I am indebted to Mrs. B. C. D[illegible] for technical assistance and to Mr T. F. J. Howson and Mr D. G. Papworth for help with the statistics.

Summary

A method for obtaining the absolute number of each type of marrow cell in experimental animals is described in detail. Absolute cell counts are given for the bone marrow and blood of two separate series of rats, one of 40 and the other of 41 animals. Some of the secular variations in cell counts coincided with a change in animal husbandry which might have entailed changes in the genetic constitution of the strain. These changes emphasize the need for frequent observations on control animals during haematological experiments. The femur, tibia and humerus contained similar numbers of nucleated cells but the marrow of the humerus contained fewer non-nucleated red cells. The cell content of marrow of a bone on one side was the same as that in the same bone on the opposite side. Exsanguination did not reduce the amount of blood contaminating samples of bone marrow.

Résumé

Description d'une méthode permettant d'obtenir des nombres absolus des différents types cellulaires de la moelle d'animaux de laboratoire. Puis les nombres absolus de cellules de la moelle et du sang de deux séries différents de rats (40 resp. 41 animaux) sont rapportés. Certaines modifications de ces nombres au courant du temps correspondent à des modifications des conditions d'élevage, qui pourraient modifier la structure génétique de la souche. Ces modifications démontrent la nécessité d'examens fréquents chez des animaux de contrôle pendant des études hématologiques. Fémur, tibia et humérus contiennent le même nombre de cellules nucléées, alors que la moelle de l'humérus contient moins de cellules anucléées de la série érythrocytaire. Le nombre cellulaire de la moelle des os correspondants des deux côtés est identique. Une saignée ne produit pas de diminution de la quantité de sang dans la moelle.

Zusammenfassung

Es wird eine Methode zur Feststellung der absoluten Zahlen der einzelnen Zelltypen des Knochenmarkes bei Versuchstieren beschrieben. Die absoluten Zellzahlen von Knochenmark und Blut zweier separater Serien von Ratten (40 resp. 41 Tiere) werden mitgeteilt. Veränderungen der Zellzahlen im Laufe der Zeit fielen zusammen mit Änderungen der Zuchtbedingungen, die die genetische Struktur des Stammes beeinflussen konnten. Solche Veränderungen unterstreichen die Notwendigkeit häufiger Stichproben an Kontrolltieren im Laufe hämatologischer Versuche. Femur, Tibia und Humerus enthielten gleiche Zahlen kernhaltiger Zellen, aber das Knochenmark des Humerus wies weniger kernlose rote Elemente auf. Der Zellgehalt des Knochenmarkes war in entsprechenden Knochen beider Seiten gleich. Entblutung führte nicht zu einer Verminderung der Blutbeimengung zum Knochenmark.

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Xth Congress of the International Society of Haematology

Stockholm August 30—September 4 1964

Preliminary Programme

Main Themes. A. Myeloproliferative diseases – leukokinetics, chromosome abnormalities and present status of chemotherapy – B. Production and destruction of haemoglobin and red cells. – C. Vitamin B_{12} and folic acid. – D Gamma globulins synthesis, identification and clinical correlations. – E. Aplastic anaemia. – F Iron metabolism. – G Hereditary coagulation disorders. Fibrinolysis. – H Lymphoid tissue with special reference to the thymus. – I Concepts of autoimmunity and their application in haematology – K. Biochemistry of red cells, leukocytes and platelets.

Symposia will be arranged on various subjects. The following have already been settled Special paediatric problems Congenital bone marrow hypoplasia. The development of haemostatic mechanisms. Erythroblastosis Prevention of intrauterine death. Humoral regulation of haematopoiesis (erythropoietin) Abnormal haemoglobins.

The second circular containing further information can be obtained from the Congress Bureau P O Box 638, Stockholm 1 Sweden.

Final application for membership March 1 1964 Final application to present paper April 1 1964

Erratum

Attention is drawn to the fact that an unfortunate mistake occurred in the paper by E. Robinson and D Nielsen entitled "The Direct Antiglobulin Consumption Test after Radiotherapy and Chemotherapy published in ACTA HAEMATOLOGICA Vol. 30 No. 3 pp. 138–143 (1963)

I Table I page 139 item 9 should read Chordoma N_2H" and not N_2N

Acta haemat. 31 65–78 (1964).

Medical Unit, University College Hospital Medical School London

Studies in Congenital Non Spherocytic Haemolytic Anaemias with Specific Enzyme Defects

By A. J Bowdler and T A. J Prankerd

The major forms of congenital haemolytic anaemia have been readily identified by distinctive clinical features, racial associations and red cell morphology and physical characteristics. Apart from the haemoglobinopathies, the most widely recognized form of such diseases is hereditary spherocytosis, which with few exceptions shows a surprisingly stereotyped pattern, and it was by contrast with this that a small group of congenital anaemias without spherocytosis was distinguished (7 11 5 8, 9 6) There are many variants among the minor haemolytic disorders, but the congenital non-spherocytic haemolytic anaemia complex contains a group having in common 1) red cells which differ little from normal in morphology or when freshly drawn in osmotic resistance, 2) no dramatic response to splenectomy 3) a familial tendency often difficult to elucidate and 4) an ethnic background almost invariably of North European origin

Within the group so defined, there are many variable features, and this is especially so with regard to the non morphological aspects of the red cell Selwyn and Dacie (18) suggested possible differences in red cell metabolism by showing that on incubation cells from 2 cases differed from normal only in failing to reduce autohaemolysis on the addition of glucose (type I) while in 2 other instances there was a marked increase in autohaemolysis (type II) This variability has now been extended by the detection of a variety of enzyme deficiencies. The relationship between such demonstrable defects and the shortening of the red cell life-span remains to be demonstrated and whether these are causal or incidental is unknown. The significant features, aetiologically are still uncertain, and the investigation of the present cases, which

illustrates three specific red cell enzyme deficiencies, is intended to complement the growing but as yet largely unorganized, approach to such understanding

Methods of Investigation

Heparinized blood taken by venipuncture was used except where otherwise stated. Standard methods for routine haematological investigations were adopted from DACIE (4)

Glucose utilization and lactate production were estimated before and after incubation of whole blood samples at 37° by the methods of SEIFTER et al. (17) and BARKER AND SUMMERSON (1)

Phosphate esters separated by trichloracetic acid extraction from washed red cells were assayed by the method of PRANKERD AND ALTMAN (15)

For enzyme studies the red cells were separated from white cells by threefold washing in saline. For estimations on leucocytes, buffy coat suspensions were used. Glucose-6-phosphate dehydrogenase ctivity was estimated by an adaptation of the method of HORECKER AND SMYRNIOTIS (14) Pyruvic kinase ctivity was estimated by modification of the method of BUCHER AND PFLEIDERER (3)

Autohaemolysis estimations were made by the methods of DE GRUCHY et al. (6)

Isotope investigations of red cell survival were made by labelling the subjects red cells with Na_2 ^{51}CrO of high specific activity washing the red cells free of unabsorbed isotope with sterile isotonic saline (10) Surface counting after injection of the labelled cells was made with collimated scintillation counter (2) The $T\frac{1}{2}Cr$ was estimated by regressing the log of the percentage activity remaining against time, determining the best fit of time for the 50% survival.

Case Reports and Results

Case 1 Glucose-6-phosphate dehydrogenase deficiency (G6-PD)

R. L. In childhood, icterus was first observed t birth, but this subsided spontaneously within few days, and no other abnormality was found until jaundice reappeared at the age of 11 months. There followed series of episodes of fever and listlessness lasting 3 to 4 days, each with jaundice pallor and dark urine. At the age of 4 he was examined in one of these ttacks the skin and conjunctivae were icteric the liver was just palpable and the spleen was felt 3 cm below the costal margin. Investigation t this time showed haemoglobin 10.8 g per 100 ml, red cells 3.3 m per mm³ reticulocytes 12 and white cells 12,000 per mm Red cell absolute values were MCV 106 u³ MCD 8.0 μ, MCHbC 29 1% Osmotic haemolysis began in 0.45 % saline and was complete in 0.39% The direct antiglobulin reaction was negative A Price-Jones curve showed peak t red cell diameter of 7.75 μ and secondary flat peak t 8.26 to 8.5 μ. Bone marrow smear was cytologically normal, but showed very activ myeloid and erythroid proliferation. The van den Bergh reaction gav direct weak positive result, with the indirect reaction 5.6 units. Plasma flocculation tests were negativ Plasma protein estimation showed albumin 5.2 gm % and globulin 2 4 gm% Serum alkaline phosphatase was 6 0 K.A. units. The urine showed urobilin in excess. Occult blood tests, Wassermann reaction and radiography of the chest, skull and limbs showed no bnormality

Between ttacks the haemoglobin was found to vary between 8.2 and 11 1 gm per 100 ml Splenectomy was performed at the age of 4½, but this did not improve the anaemia during the subsequent three years the haemoglobin level was found to be

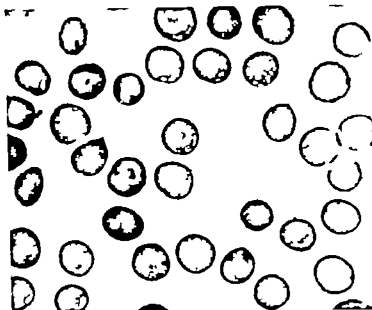


Fig. 1. Blood smear from patient R. L. (G6PD deficiency). (× 1000)

between 8.9 and 9.5 gm per 100 ml, the reticulocytosis between 6 and 20% and the patient continued to have occasional attacks of jaundice with fever.

Symptoms were few during the subsequent years of childhood and there was virtually no restriction of activity. At the age of 18 the patient was reassessed. There were no significant complaints, and no evidence of exertional dyspnoea, lassitude or malaise was elicited. On examination, the patient was definitely jaundiced, and there was some cutaneous and mucosal pallor; apart from the splenectomy scar the abdomen was normal, and there were no other physical signs.

Investigation showed haemoglobin 11.2 gm per 100 ml, red cells 3.7 m per mm³, white cells 7,000 per mm³ and reticulocytes 8.2%. Red cell absolute values were MCD 8.0 μ, MCV 107 μ³ and MCHC 28%. A blood smear showed moderate anisocytosis, with slight polychromasia and hypochromasia (fig. 1). There were no Heinz bodies demonstrable. Serum bilirubin was 10.9 mg%. Alkali denaturation showed HbF 3.5%; no abnormal haemoglobins were detected electrophoretically.

Family studies: The patient's parents were examined haematologically when he was 4 years old; the results are recorded in table I.

Autohaemolysis. The results of these studies are shown in table II. No increase in haemolysis was observed in two of the patients' relatives, but his own autohaemolysis was moderately increased at 24 hours and diminished only slightly by the addition of glucose and adenosine.

Metabolic studies: Estimation of glucose utilisation and lactate production were normal in R. L. and one of his relatives and analysis of phosphate ester composition was also normal, as were the red cell sodium and potassium concentrations (table III).

Enzyme studies. The activity of glucose-6-phosphate dehydrogenase was approximately 20% of that found in normal red cells, but estimation of this enzyme in R. L.'s leucocytes showed normal activity. Pyruvic kinase activity was normal (table IV).

Table I

Haematological data on patients and relatives.

Deficiency	Haemoglobin per 100 ml	Reticulocytes per 100 R.b.c.	Fresh Osmotic fragility Begins	Fresh Osmotic fragility Complete	Incubated Osmotic fragility Begins	Incubated Osmotic fragility Complete
			% saline			
G6PD I						
R. L.	10.9	37	0.45	0.35	0.53	0.40
G. L. (Father)	15.3	0.7	0.45	0.25	0.55	0.40
A. L. (Mother)	13.7	0.9	0.45	0.25	0.55	0.33
L. C. (Sister)	12.5	1.5	0.45	0.25	0.55	0.35
P. K. II						
D. V.	7.8	15.5	0.45	0.21	0.65	0.27
A. V. (Father	14.4	1.6	0.45	0.30	0.63	0.33
M. (Mother)	13.9	1.0	0.45	0.33	0.69	0.30
B. (Brother)	14.9	1.1	0.45	0.30	0.60	0.33
3-PGAM III						
A. F	12.5	5.6	0.51	0.24	0.90	0.30
E. F (Father	12.7	6.6	0.57	0.21	0.90	0.30
M. F (Mother propositus also affected	14.4	0.5	0.45	0.30	0.65	0.33

Table II

Results of autohaemolysis studies with different substrates in members of each family

		NR	Glucose	Substrate Added Adenosine	ATP
Normal		1.2–[illegible]	0.1–0.5	0.1–0.3	0.1–0.5
I	R. L.	6.5	6.0	3.4	3.4
	G. L.	1.8	0.5		
	A. L.	2.1	0.8	0.2	0.4
II	D. V.	15	10	12	5
	F	1.3	0.1		
	M	1.8	0.5		
III	A. F	12	1.9	0.8	6
	E. F	16	2.2	4.1	4.0

Figures represent per cent haemolysis at 24 hours.

Radioisotope studies: Labelled red cells, re-introduced into the patients' circulation, disappeared rapidly in apparently exponential fash[illegible] T½Cr being calculated as 4.25 days, corresponding to a mean red cell lifespa[illegible] six days (fig. Surface counting showed [illegible] ty in all t[illegible], and suggested that destruction of [illegible] ng diffusely [illegible] l system (fig. 2b

When labelled [illegible] he activity left the circulation w[illegible] was [illegible]

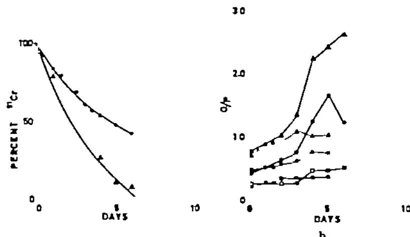


Fig 2a. Survival of ^{51}Cr-labelled autogenous red cells in patients R. L. before and after taking 5 g ascorbic acid daily
No ascorbic acid. 5 g ascorbic acid daily

Fig 2b. ^{51}Cr organ-precordium (O/P) activity after the injection of autogenous ^{51}Cr labelled red cells in patient R. L. before and after taking 5 g ascorbic acid daily
Without ascorbic acid With ascorbic acid

Liver-precordium ratio
Lumbar spine-precordium ratio
Spleen-precordium ratio

rate over the splenic area. The T½Cr was 2.9 days indicating red cell lifespan of 4.2 days (fig. 3 and b). This was significantly reduced compared with the lifespan of the cells in the donor circulation ($t = 2.37$ $p < .05$) The survival study was repeated with the recipient taking primaquine. There was no significant difference in the red cell survival (= 0.75) Surface counting showed that there was accumulation of activity at all the sites investigated (spleen, liver and lumbar spine). In view of this failure of primaquine to shorten the survival of the enzyme defective cells, the survival of the patient' cells was followed whilst he was taking 5 g ascorbic acid daily in the hope that this might provide an alternate redox system to glutathione However on this regime there was shortening of the patient' red cell survival.

Case 2. Pyruvic kinase deficiency (PK)

D F was 5 years old when first studied. At birth he had been jaundiced and there had been evidence of Rh incompatibility between mother and child. After an exchange transfusion the jaundice recurred and further transfusions were required on account of recurring anaemia. At the age of two splenectomy was performed with some diminution in the transfusion requirement and subsequently the haemoglobin concentration stabilised between 7 4 and 8.29 gm/100 ml. There was no family history of anaemia, and he had one healthy brother

Examination revealed slightly jaundiced normally developed child with a liver showing painless firm enlargement 4 cm below the costal margin. Blood examination showed haemoglobin 7.8 gm ., and smear showed macrocytosis and polychromasia (MCHC 33%; MCV 102 μ^3) (fig 4). Heinz bodies were present ad there was ready

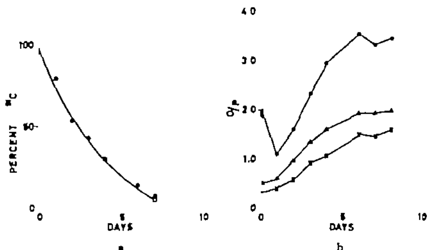


Fig 3a. Survival of ⁵¹Cr labelled red cells from patient R. L. injected into normal compatible recipient before and after taking primaquine
Without primaquine. With 30 mg primaquine daily

Fig 3b. ⁵¹Cr organ-precordium (O/P) activities in recipient taking 30 mg primaquine daily after the injection of R. L. red cells.
Spleen-precordium ratio. Liver-precordium ratio. Lumbar spine ratio.

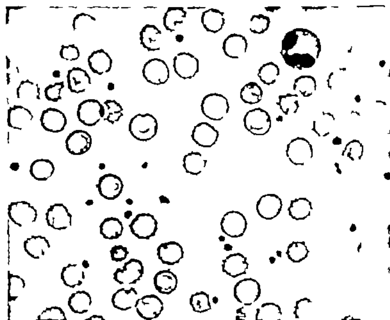


Fig 4 Blood smear from patient D \ (PK deficiency) (× 600).

Table III
Results of metabolic studies in members of each family

	Glucose utilisation	Lactate formation	ATP	ADP	IP	2,3 PGA	Na	K
				Phosphate ester content				
	(μ mol /ml cells/hour)			μg P./ml cells			mEq/L cells	
Normal	1.8–2.2	4.0–4.7	55–70	10–25	35–50	150–180	10–19	95–110
Case I								
R. L.	2.1	4.4	68	12	41	184	15	110
G. L.	2.0	4.0	59	15	40	164	17	102
Case II								
D. V	0.5	1.2	27.5	18	63	225	35	80
A. V	1.9	4.4	61	15	46	170	15	116
M.	2.2	4.5	71	10	51	161	19	104
Case III								
A. F	1.6	4.0	45	20	64	84	15	105
L. F	1.6	3.8	50	25	71	65	17	99

formation of crenated cells. Reticulocytes numbered 15.5/100 red cells. White cells and platelets were normal in number and appearance. The direct antiglobulin test was negative. Paper electrophoresis of haemoglobin showed no abnormality. The foetal haemoglobin concentration was 1.2%. Serum bilirubin was 2.3 mg/100 ml, with no directly reacting pigment. Red cell osmotic fragility showed the presence of about 60% of cells with increased resistance, with no increased fragility of fresh or incubated cells.

Autohaemolysis: The results of these studies are shown in table II. There was markedly increased autohaemolysis which was only partly corrected by glucose, adenosine and ATP. Results in the relatives were normal.

Metabolic studies showed considerable reduction in glucose utilisation and also in lactate production suggesting severe impairment of glycolysis (table III). Analysis, of red cell phosphate esters showed an abnormal pattern with low values of ATP normal values of ADP and inorganic phosphate and high values of 2,3-PGA. The sodium concentration in the cells was higher than normal with reduction in potassium concentration. In the relatives studied all the data were within normal limits.

Enzyme studies. Red cell glucose-6-phosphate dehydrogenase activity was slightly increased in DV but there was severe impairment of pyruvate kinase activity (table IV). All the relatives had normal G6PD activity in their red cells, but estimations of pyruvic kinase in relatives showed reduced activity in all, of pattern to suggest that the parents were heterozygotes.

I DV the leucocyte PK activity was estimated and found to be within the same range of activity as in the leucocytes of three normal individuals.

Red cell survival studies showed T ½ Cr to be 8 days corresponding to mean life-span of approximately 14 days for autologous cells in the patient' own circulation.

Case 3. [illegible] deficiency (2,3-PGAM)

H F was first noticed to be jaundiced at 3½ years, during an ttack of fever with bdominal pain. At this time splenomegaly was found (1 cm below costal margin)

Table II
Results of enzyme assays in red cells and leucocytes of members of each family

		Red Cells†		Leucocytes*	
		G6PD	PK	G6PD	PK
Normal		1 1 1.9	0.18–0.31	250–310	320–480
I	R. L.	0.3	0.27	270	—
	G. L.	1.6	0.30	—	—
	A. L.	1.9	0.20	—	—
	I. C.	1.6	0.24	—	—
II	D. A.	2.3	0.03	—	340
	M.	1.6	0.09	—	—
	F.	1.8	0.12	—	—
	D.	1.4	0.10	—	—
III	F	1.4	0.21	—	—
		1.2	0.21	—	—

*Units per 10^9 Wbc.
† Δ O D /min/ml cells (25°)

but there was no hepatic enlargement. Investigations at this time showed Hb. 10.8 gm/100 ml, reticulocytes 3.7 normal osmotic fragility, a negative antiglobulin test and hyperplastic bone marrow.

Attacks of fever and abdominal pain recurred and jaundice persisted. Haemoglobin levels fluctuated between 8.3 and 10.2 gm% and one transfusion was given.

At the age of 12 he was re-investigated. At this time he appeared mildly jaundiced and the spleen was just palpable below the costal margin. Investigations at this time showed haemoglobin 12.5 gm/100 ml, red cells 4.0/mm³, white cells 3,700/mm³ and reticulocytes 5.6%. Red cell indices were MCD 7.0 μ, MCV 90 μ³, MCHC 34%. A blood smear showed moderate anisocytosis and polychromasia, and there were no Heinz bodies demonstrable. Serum bilirubin was 2.1 mg/100 ml, with no direct reaction. Alkali denaturation showed Hb. F 1.1 but no abnormal haemoglobins were detected electrophoretically.

The patient's father had had splenectomy for "haemolytic anaemia" at the age of 30 years, having been intermittently jaundiced since the age of 7 years. A paternal aunt was said to have been anaemic. Haematological data of the parents are summarised in table I.

Following splenectomy the patient remained well, but the jaundice still continued. Three years later his haemoglobin was 13 gm/100 ml and during this period had not fallen below this figure. At this time reticulocytes were 3.1% and serum bilirubin 2.1 mg/100 ml.

Autohaemolysis. Autohaemolysis of red cells of both the patient AF and his father were considerably increased on incubation and reduced by the addition of glucose and adenosine and also by ATP (table II).

Metabolic studies. These showed reduced utilization of glucose and production of lactate by A. F. cells with very low contents of 2,3-PGA in both his cells and those of his father. The content of ATP was slightly reduced, but there was also an increase in fructose 1–6 diphosphate. The sodium and potassium contents were within normal limits (table III).

Table V

Results of phosphate ester content of red cells in affected members of each family before and after incubation without glucose + NaF and after the addition of glucose (0.01 M).

	Before incubation without glucose		After incubation with NaF for 1 h	
	F16P	23PGA	F16P	23PGA
Normal range	10–15	150–180	30–51	200–250
R. L.	12	184	24	192
D. V	37	225	31	233
A. F	54	84	51	76
E. F	43	115	57	88

	After 3 h incubation			
	No glucose		Glucose added for ½ h	
	F16P	23PGA	F16P	23PGA
Normal range	4–6	100–140	8–14	140–200
R. L.	3	106	13	198
D. V	6	80	25	1.0
A. F	15	32	46	58
E. F	17	28	37	28

Figures represent µg P per ml cells.

Enzyme studies. Estimations of glucose-6-phosphate dehydrogenase in these patients were normal (table IV). The low levels of 2,3-PGA are not in keeping with those found in the patient with pyruvic kinase deficiency and to discover the cause of the low 2,3-PGA content, tests were made to assess its rate of synthesis. To do this glycolysis was blocked below the formation of 2,3-PGA by incubation with sodium fluoride. This normally leads to an increase in cell content of 2,3-PGA until a new equilibrium is reached and glycolysis ceases. In this case there was only very minimal increase in 2,3-PGA but an increase in fructose 1,6 diphosphate. These findings (table V) strongly suggested a defect in synthesis of 2,3 diphosphoglycerate. A further study was also made in which A. F's red cells were depleted of 2,3-PGA by incubation in saline without glucose for 4 h. When the 2,3-PGA content had fallen to about 40% of its original value glucose (0.01 M) was added to the suspension and incubation continued for a further hour. In normal cells there was a rapid increase in 2,3-PGA content as glycolysis resumed, but in A. F's cells virtually no 2,3-PGA reaccumulated although glycolysis was occurring at a reasonable rate.

Red cell survival studies showed the mean survival of the patient's cells in his own circulation to be approximately 40 days (T½ ^{51}Cr = 18 days) after splenectomy

Discussion

Attention was focused on the group of congenital haemolytic disorders under consideration by DACIE et al. (5) who distinguished them from the more specific disorder hereditary spherocytosis. This distinction can be made in most cases without difficulty and is of clinical importance because of the differences in natural history and especially the response to splenectomy. The main factor in producing haemolysis in these disorders is probably an intrinsic

chemical abnormality of the red cells and SELWYN AND DACIE (18) found non-specific evidence of this in autohaemolysis studies in 4 cases. Other evidence of erythrocyte metabolic abnormality has been provided by PRANKERD (13) and ROBINSON et al. (16) from a study of red cell phosphate esters. In the last few years evidence of specific enzyme deficiencies has emerged notably of glucose-6-phosphate dehydrogenase (12 19 21) and pyruvate kinase (20)

This report describes cases from three families with congenital nonspherocytic haemolytic anaemia, in which different, specific enzyme defects were demonstrable in the red cells. Autohaemolysis studies *in vitro* showed that the glucose-6-phosphate dehydrogenase deficient cells in case 1 behaved in the type I fashion, while the cells from cases 2 and 3 behaved as the type II variant, showing marked autohaemolysis on incubation, which was partly diminished by the addition of glucose and restored virtually to normal levels with ATP These findings are comparable to those of TANAKA AND VALENTINE and MIWA (20) who found that pyruvate kinase deficient cells showed the type II pattern of autohaemolysis. These differences correlate with the finding of abnormal patterns of red cell phosphate esters and defective glucose utilization by red cells in cases 2 and 3 although these were substantially normal in case 1 It is clear that in the two former cases, the enzyme defect involved a block in the main pathway for red cell carbohydrate metabolism, while in case 1 the defect was in a subsidiary cycle the pentose phosphate pathway

The family showing pyruvate kinase deficiency demonstrated a genetic transmission of this abnormal enzyme in keeping with the recessive pattern described by TANAKA et al. (20) those members of the family showing intermediate enzyme activity having no evidence of haemolysis.

This enzyme controls one of the terminal reactions of the Embden Myerhoff sequence in which a synthesis of ATP and ADP occurs with the dephosphorylation of phospho-enol pyruvate to pyruvate. It is therefore a necessary step in the provision of chemical energy within the cell and as such any impairment of its activity may be expected to lead to a fall in cell ATP and a disturbance of cell electrolytes and of cell viability (PRANKERD 1951) An increase in 2 3-diphosphoglycerate would be expected on the basis of a build up of substrates proximal to the enzyme defect the

impairment of glucose consumption and lactate production would be a logical result of the block in glycolysis.

None of these features was observed in the patient's cells which were deficient in G6PD (case 1) and it is noteworthy that in this patient the cell electrolytes and ATP contents were normal, implying a different haemolytic mechanism at work.

It is interesting that TANAKA et al. (20) reported normal leucocyte pyruvic kinase activity in their cases and this appeared to be the case in the patient studied in this family. This finding would seem to indicate that the disease is not due basically to one enzyme deficiency as the inheritance of this would be expected to affect all tissues. A similar situation was observed in the family with G6PD deficiency, the leucocyte activity of this enzyme also within normal limits. Situations such as these, in which only one tissue appears to be affected are more likely to result from the inheritance of an inhibitory factor which becomes active only in some specialized and highly adapted cells, unless separate genes control the same enzyme in different tissues.

The third family showed indirect evidence of a deficiency of the enzyme diphosphoglyceromutase. This consisted of the demonstration of low levels of 2,3-PGA in the red cells and of an inability to synthesize this compound when the cells were incubated with sodium fluoride, or after exhaustion of 2,3-PGA, with glucose. Sodium fluoride blocks glycolysis in normal cells at the step in which enolase is active, with the result that glycolysis continues for a limited period during which intermediates, formed before 2 phosphoglycerate, accumulate in the cells. This leads, particularly in normal cells, to an accumulation of 2,3-PGA. Its failure to accumulate in these patients' cells coupled with the increase in content of fructose 1-6, diphosphate, strongly suggests an inability to convert 1,3-PGA to 2,3-PGA. Corroborative evidence was the failure to synthesise 2,3-PGA after its exhaustion by incubation without glucose, even though the cells were at that time when glucose had been added utilizing glucose.

The net result of these metabolic abnormalities on the red cell vary. In two abnormalities, PK and 2,3-PGA mutase deficiency the defects result in a fall in cell ATP and potassium content which may reasonably be adduced as a cause of their failure to survive in the circulation. In the case of G6PD deficiency no fall in ATP content occurred and a more subtile process must be at work. The

normally relatively inactive state of the pentose phosphate pathway suggests that the enzyme deficiency itself is unlikely to be a major factor in causing the cells to survive for such a short interval after discharge into the circulation.

The pattern of red cell destruction *in vivo* was investigated in detail only in case 1 but it is remarkable that the most profound shortening of the red cell life was to be found in this instance, in which metabolic abnormalities were the most difficult to demonstrate. The pattern of red cell destruction was close to a purely exponential loss of cells from the circulation and as has been found by others (6) the loss was more rapid with cells cross-transfused to a compatible subject. It is noteworthy that primaquine did not accelerate the cell destruction in the recipient (fig 3) but ascorbic acid given in doses of 5 gm daily produced a significant acceleration in red cell destruction in the patient. These studies were made long after splenectomy and it is thus not possible to define what part this organ originally played in producing the anaemia, but it seems clear that even after splenectomy very profound shortening of the red cell life is present and that the reticuloendothelial system is involved diffusely in the destructive process.

It is clear therefore, that the heterogeneity of this group of diseases is very considerable, and although this has been thought to hold more strongly for the type I cases, it is now clear that the type II cases also show variability in the discoverable defects. In the latter consequent abnormalities in the glycolytic mechanism suggest a further possible step in the sequence of events from the inherited enzyme defect to the premature destruction of the cells, but this is still far from elucidation in the type I form of the disease

Abbreviations: ATP = adenosine triphosphate 2,3-PGA = 2,3-diphosphoglycerate; F 1 6-P = fructose 1,6-diphosphate; IP = inorganic phosphate.

Summary

Three types of enzyme defect in the red cells of northern Caucasian families are described associated with congenital nonspherocytic haemolytic anaemia. The enzymes involved are pyruvic kinase glucose-6-phosphate dehydrogenase and 2,3-diphosphoglyceromutase. Measurements of phosphate esters in the red cells were made together with glucose utilization and lactate production. Enzyme assays were also made in leucocytes.

Zusammenfassung

Es werden drei Arten von Enzymdefekten der Erythrocyten beschrieben, die in nordkaukasischen Familien bei kongenitaler nichtsphärocytärer hämolytischer Anämie

vorkommen. Bei diesen Enzymen handelt es sich um Pyruvatkinase, Glukose-6-phosphat-Dehydrogenase und 2,3-diphosphoglycerat In den Erythrocyten wurden die Phosphatester der Glukoseverbrauch und die Laktatproduktion bestimmt. Enzymbestimmungen wurden auch in den Leukocyten vorgenommen.

Résumé

Description de 3 types de défauts de ferments érythrocytaires observés chez des familles du canton du nord, avec une anémie congénitale hémolytique non sphérocytaire. Il s'agit de la kinase pyruvique, de la déhydrogénase du glucose-6-phosphate et de la 2 3 diphosphoglycérate Les esters phosphoriques, la consommation de glucose et la production du lactate des érythrocytes ont été déterminés et parallèlement, les ferments leucocytaires.

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Three types of enzyme defect in the red cells of northern Caucasian families are described associated with congenital nonspherocytic haemolytic anaemia. The enzymes involved are pyruvic kinase, glucose-6-phosphate dehydrogenase and 2,3-diphosphoglyceromutase. Measurements of phosphate esters in the red cells were made together with glucose utilisation and lactate production. Enzyme assays were also made in leucocytes.

Zusammenfassung

Es werden drei Arten von Enzymdefekten der Erythrozyten beschrieben, die in nordkaukasischen Familien bei kongenitaler nichtsphärozytärer hämolytischer Anämie

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Uptake of Fe^{59} by Acid Insoluble Elements of Normal and Thalassaemic Haemolysates. In vitro Studies

By B. Malamos, E. Gyftaki and Ch. Proukakis

A number of papers have been published in the past dealing with the problem of haemoglobin synthesis in thalassaemia. The constantly observed hypochromia of the red cells, inspite of the normal or increased time of their maturation (1) and the presence of abundance of iron, points to a disturbance in haemoglobin synthesis.

By incubating blood in vitro in the presence of Fe^{59} and/or glycine-C^{14} Bannerman et al. (2) reported that haem synthesis in thalassaemia per immature red cell is reduced as compared to other haemolytic anaemias, while globin synthesis is normal. In a previous paper (10) we reported that the iron uptake per reticulocyte (in vitro) is much higher in thalassaemic patients than in normals.

The purpose of the present study is to report on the iron incorporated in vitro in the TCA* precipitable fraction of the haemolysate of thalassaemic patients and subjects with thalassaemia trait and to compare this incorporation with that of normal haemolysate It is taken for granted that in all cases iron incorporation takes place only in the immature red cells.

Material and Methods

Cases studied

Thirty persons were studied 10 normals, 10 thalassaemic patients and 10 carriers of the thalassaemia trait. As normals are considered persons with normal blood picture and free of pathologic conditions which could influence the haematopoietic activity As carriers are considered persons with anisocytosis, poikilocytosis, microcytosis, MCH < 23 μμg and increased Hb A_2, with no additional conditions which could influence the

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* *Abbreviations:* TCA trichloracetic acid; MCH mean corpuscular haemoglobin (in μμg); HCT haematocrit; IRC immature red cells (reticulocytes + erythroblasts); PCV packed cell volume.

Table I

Normal subjects.

No.	Sex	Age years	HCT %	Hb gr %	Red cells 10^6	Immature red cells %
1	M	33	45	15.2	5420	1.20
2.	F	36	41	13.3	4180	0.70
3	F	17	43	14 1	4890	0.90
4	F	54	42	13.1	4600	1.30
5.	F	30	45	15.4	4960	1.00
6.	M	38	43	14.3	4740	0.80
7	F	43	41	14.4	4440	1.20
8.	F	28	45	14.8	4810	1.30
9	M	59	44	14 4	4690	1 60
10	M	39	46	14 8	4810	1.30

Table II

Thalassaemia trait carriers.

No.	Sex	Age, years	HCT %	Hb gr %	Red cells 10^6	Immature red cells %	Hb. A2 increased	MCH
1	F	40	38	13.1	5890	1.80	+	22
2.	M	23	44	13.7	6450	0.80	++	21
3.	M	31	43	13.5	6270	3.10	+	21
4	M	30	40	12.9	5880	2.80	++	22
5.	F	40	40	11.6	5850	1 40	++	20
6.	F	17	39	11.0	5380	1.60	++	20
7	F	35	37	11.6	5120	0.80	+	23
8.	F	40	39	11.5	5360	0.90	+	21
9	M	48	43	13.5	6300	1.50	+	21
10.	M	54	47	14 0	6960	1.90	++	20

haematopoietic activity As thalassaemic patients are considered persons with the clinical and laboratory findings of thalassaemia major

The sex, ge and haematological data are shown in tables I II and III

Methods

The method used for the incubation of blood with Fe^{59} is that described by BELCHER AND COURTENAY (5) with minor modifications

Thirty-two ml of blood were taken with heparinized syringe and transferred immediately into 500 ml Erlenmayer flask. Eight ml of 1% glucose in saline and 5 μc of Fe^{59} were added. The time which elapsed from the withdrawal of the blood to the beginning of the incubation was 20 to 45 minutes.

Determinations of red cells, HCT serum iron and immature red cells were done on the same sample and 0.5 ml was kept as standard. The remaining blood was incubated 37 C in the presence of oxygen. After 1 4 and 6 hours incubation two separate samples were withdrawn (one of 0.5 ml and the other of 10 ml). They were washed four times with saline and centrifuged at 4 C. The radioactivity of the standard and of the 0.5 ml samples were measured at this point.

2 μc per ml, containing less than 1 μg of iron, in 1% (W/V) sodium citrate solution.

Table III
Thalassaemia major

No.	Sex	Age, years	HCT %	Hb g %	Red cells 10^3	Hb. F %	Immature red cells %	Recently transfused
1	M.	5	26	6.5	3900	82	4.51	—
2.	M.	28	9	2.7	1410	67	1.70	—
3.	F	6	25	7.0	3120	82	5.21	+
4.	F	11	15	4.3	2450	22	2.92	—
5.	M.	18	20	6.8	2550	48	4.84	+
6.	F	4	15	4.8	1880	15	0.97	+
7	M.	6	12	3.8	1760	33	4.63	+
8.	F	4	25	6.4	2430	20	3.46	+
9.	M.	2.5	13	4.7	2060	38	3.70	+
10.	M.	34	19	6.4	2440	26.5	4.52	+

The mean iron uptake per immature red cell (in μg) was estimated using the formula

$$\frac{W}{S} \times \frac{\text{plasma iron concn. } (\mu g/ml) \times (100\text{-PCV } (\%))}{\text{red cell count (cells/mm}^3) \times \text{IRC count } \%} \times 10^{-4}$$

where W = Fe^{59} content of washed ed cell sample 0.5 ml (counts/m) and
S = Fe^{59} content of standard (counts/m)

The red cells of the 10 ml sample were haemolysed with distilled water and were allowed to stand for 15 min at 4 C. The stroma was separated according to the method of Tishkoff et al. (14).

The solutions employed to wash the stroma were added to the stroma free haemolysate and the total volume was measured. Solid trichloracetic acid (TCA) was added to final concentration of 5% and the precipitate was taken by filtration. The radioactivity of the TCA precipitable fraction was measured in well-type scintillation counter operated in conjunction with single-channel pulse-height analyser (Nuclear Chicago No. 132)

Results

The value of the iron located in the TCA precipitable fraction is expressed in two ways a) as per cent of iron taken up by the TCA precipitable fraction of the immature red cells and b) as absolute value of iron in the same fraction estimated in μg of iron per immature red cell. Knowing the per cent of Fe^{59} incorporated in the TCA precipitable fraction of the haemolysate as well as the mean iron uptake per immature red cell, one can estimate the average amount of iron present in the TCA precipitable fraction of each I R.C.

In calculating the amount of iron incorporated in the TCA precipitable fraction, we take it for granted that intracellular isotope dilution in normal cases is not existent at least not to a significant extent (11) However even when one accepts that isotope dilution does occur in thalassaemic cases, it would mean that the amount of iron incorporated is actually more than what we calculate in the

Table IV

Results in normal subjects.

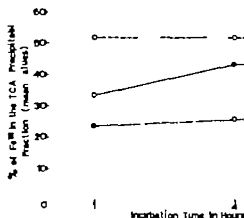
No.	Incubation time in hours	Iron uptake in μg 10^{-10} per immature red cell	% of Fe^{59} in the TCA precipitable fraction	Amount of iron in μg 10^{-10} per immature red cell in the TCA precipitable fraction
	1	2.64	47.0	1.24
1	4	4 15	59.3	2.46
	6	5.49	49.3	2.71
	1	5.30	38.1	2.02
2	4	6.21	40.3	2.50
	6	8.04	54.5	4.38
	1	[illegible]19	35.9	0.78
3	4	4 18	54.8	2.29
	6	3.94	52.3	2.06
	1	2.92	36.5	1.07
4	4	3.65	41 1	1.50
	6	4.31	36.2	1.56
	1	1.06	30.6	0.32
5	4	1.84	47.0	0 86
	6	2.68	50.2	1.35
	1	3.06	21.9	0.67
6	4	4.42	29.6	1.31
	6	4.36	31 7	1.38
	1	1 72	42.5	0.73
7	4	3.13	53.0	1.66
	6	4.83	44.9	2.17
	1	1.93	31.3	0.66
8	4	2.44	31.5	0.77
	6	4.31	35.6	1.54
	1	0.54	32.6	0.18
9	4	0.99	43.1	0.43
	6	2.59	41.0	1.06
	1	2.02	17 4	0.35
10	4	2.82	32.5	0.92
	6	3.29	44.1	1 45
		Mean values		
	1	2.34	33.7	0.80
	4	3.38	43.2	1 47
	6	4.38	44.0	1.96

study which would of course tend to increase the differences report ed herein even more.

Tables IV V and VI show the experimental data. Table VII gives an analysis of the data, while figures 1 and 2 are a graphic representation of the same.

Discussion

The mean values of the per cent iron located in the TCA precipitable fraction at six hours (as 100 % is considered the total up-

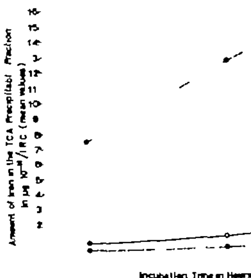


Thalassaemia Major

—— Normal Subjects

- - - Thalassaemia Trait

Fig 1



Thalassaemia Major

—— Normal Subjects

- Thalassaemia Trait

Fig 2

take of iron by the immature red cell) are for the normals 44 0 % for the thalassaemic patients 51 1 % and for the carriers 26.6 %. It is clear that in this respect there is no significant difference between normals and thalassaemic patients ($p = 0\ 1$) while there is such a difference between normals and carriers ($p = 0\ 002$)

Table I

Results in thalassaemia trait carriers.

No.	Incubation time in hours	Iron uptake in $\mu g \times 10^{-11}$ per [illegible] red cell	% of Fe^{59} in the T.C.A. precipitable fraction	Amount of iron in $\mu g \times 10^{-11}$ per [illegible] red cell in the T.C.A. precipitable fraction
	1	3.50	31.8	1.22
1	4	6.01	43.0	2.58
	6	6.87	37.5	2.58
	1	2.80	28.0	0.78
2	4	3.88	27.1	1.05
	6	3.64	46.4	1.69
	1	0.74	24.9	0.18
3	4	1.26	14.1	0.18
	6	1.81	14.5	0.26
	1	1.71	11.6	0.20
4	4	2.89	11.9	0.34
	6	3.32	11.5	0.38
	1	1.38	14.3	0.20
5	4	1.30	18.6	0.24
	6	2.05	13.0	0.27
	1	1.32	23.1	0.30
6	4	1.84	19.5	0.36
	6	2.37	17.6	0.42
	1	1.41	30.9	0.44
7	4	2.18	47.4	1.03
	6	2.76	43.7	1.21
	1	1.13	29.8	0.34
8	4	1.54	32.5	0.50
	6	2.32	34.6	0.80
	1	0.73	19.9	0.15
9	4	1.70	20.6	0.35
	6	2.23	21.5	0.48
	1	1.44	20.6	0.30
10	4	1.88	23.2	0.44
	6	2.22	25.7	0.57
		Mean values		
	1	1.62	23.8	0.41
	4	2.43	23.8	0.71
	6	2.96	26.6	0.87

In addition our data show that even though the per cent of Fe^{59} located in the TCA precipitable fraction of the haemolysate is about the same for normals and thalassaemic patients, the total amount of iron located in the same fraction in thalassaemia is approximately 8 times higher than normal (15.88×10^{-11} μg/I.R.C. as compared to 1.96×10^{-11} μg/I.R.C. for normals after 6 hours incubation). This difference is statistically significant ($p < 0.001$)

Table VI

Results in thalassaemic patients.

No.	Incubation time in hours	Iron uptake in μg 10^{-10} per immature red cell	% of Fe^{59} in the T.C.A. precipitable fraction	Amount of iron in μg 10^{-10} per immature red cell in the T.C.A. precipitable fraction
	1	14.23	65.0	9.26
1	4	30.70	66.2	20.32
	6	36.50	66.6	24.31
	1	26.84	56.4	15.14
2	4	29.93	61.6	18.43
	6	25.91	68.5	17 73
	1	11.53	61.9	7 14
3	4	29.79	53.8	16.03
	6	48.21	49.1	23.67
	1	8.45	40.6	3.43
4	4	13 36	34.9	4.66
	6	17 47	32.0	5.58
	1	9 78	32.5	3.18
5	4	11.82	43.8	5.18
	6	16.36	44.9	7.35
	1	24.32	49 1	11.94
6	4	27.77	45.1	12.52
	6	30.32	48.8	14.80
	1	6.26	53.1	3.32
7	4	12.82	52.4	6.72
	6	14 70	48.3	7 10
	1	14 78	63.6	9 40
8	4	33.83	59.0	19.96
	6	43.94	55.7	24 47
	1	15.40	48.4	7 45
9	4	34 70	57.8	20.06
	6	47 74	53.1	25.35
	1	9 43	48.8	4.60
10	4	12.98	44.7	5.80
	6	19.00	44 1	8.38
		Mean values		
	1	14.10	51.9	7 49
	4	23.77	51.9	12.97
	6	30.01	51.1	15.86

The observed difference in the iron uptake by the TCA precipitable fraction of the individual I.R.C. s between normal and thalassaemic cases may be partly due to the more immature state of the peripheral blood in thalassaemia. The expected difference should be relatively small since the more immature marrow cells incorporate only five times as much iron as normal reticulocytes do. However according to our findings thalassaemic reticulocytes incorporate in the TCA precipitable fraction eight times as much iron as

Table VII

Standard deviation of the % of Fe^{59} in the TCA precipitable fraction and of the amount of iron in $\mu g \times 10^{-11}$ per immature red cell in the same fraction.

	Normal subjects		Thalassaemia-trait		Thalassaemia patients	
Incubation time in hours	% of Fe^{59} in the T.C.A. precipitable fraction	Amount of iron in the T.C.A. precipitable fraction	% of Fe^{59} in the T.C.A. precipitable fraction	Amount of iron in the T.C.A. precipitable fraction	% of Fe^{59} in the T.C.A. precipitable fraction	Amount of iron in the T.C.A. precipitable fraction
1	33.7± 8.8	0.80±0.54	23.8± 6.3	0.41±0.34	51.9±10.3	7.49±4.04
4	43.2±10.3	1.47±0.75	25.8±11.8	0.71±0.72	51.9± 9.7	12.97±6.77
6	44.0± 7.6	1.96±0.96	26.6±13.0	0.87±0.76	51.1±10.7	15.88±8.24

the respective normals. It is apparent that the difference in maturation between normal and thalassaemic reticulocytes is of smaller order of magnitude than between reticulocytes and bone marrow erythroblasts and consequently the higher uptake could not be *accounted for solely on the basis of this difference in maturation.*

Corroborative evidence was offered by STURGEON AND FINCH in 1957 (13) who estimated the intramedullary production of haemoglobin in children with thalassaemia and found it approximately 6 times normal. Similarly ERLANDSON et al. (5) in 1958 in a more extensive study on patients with thalassaemia, observed that the amount of the synthesized haemoglobin is 1.5–5.4 times normal. An increase in erythropoietic activity of the bone marrow in 24 patients with thalassaemia has been reported by this laboratory (9). The fact that in in vivo studies the incorporation of Fe^{59} in the red cells, which represents the effective erythropoiesis, is lower than normal is generally accepted as evidence that the erythropoiesis in thalassaemia is ineffective and that there is some disturbance in haemoglobin synthesis.

TISHKOFF (15) accepts, that the fault in thalassaemia is due to an impairment of the intraerythrocytic utilization of iron. Namely the iron is incorporated into the haem but (for reasons unknown as yet) this incorporation is small. Also BANNERMAN et al. (2) express the opinion, that there is a partial block in the incorporation of iron into protoporphyrin. They report in a study similar to the present, that haem synthesis is lower than in other haemolytic anaemias. In their paper they did not compare the data from thalassaemic patients with normals, but rather from thalassaemic patients with patients of other haemolytic anaemias. It is known that different haemolytic conditions show increased haematopoietic activity over normal but this is of varying degree. Thus BOTHWELL et al. (4) found,

in acquired haemolytic anaemias of various duration and severity that the production of haemoglobin, measured by means of plasma iron turnover ranged between 3 to 6 times normal. An additional factor which possibly influences the results of such a study is the time which elapses from the withdrawal of blood to the start of the incubation Since the ability for haemoglobin synthesis by the immature red cells is impaired in in vitro studies, this time which elapses may accentuate the impairment even more.

The data of the present study indicate that there is no impaired intraerythrocytic incorporation of iron into the TCA precipitable fraction in thalassaemia, which does not necessarily mean unimpaired incorporation of iron into haemoglobin.

Tishkoff (15) expresses the view that red cells lose iron during maturation. In this respect Grinstein et al (7) investigating the subject of ineffective erythropoiesis, discuss the possible loss of haemoglobin from the immature red cell during maturation. These workers noted, after administering glycine-C^{14} to three patients suffering from thalassaemia, an increase of radioactivity in stercobilin starting 24 hours after the administration of the radioisotope reaching a maximum value the third day The greatest portion of this stercobilin does not seem to result from the breakdown of circulating haemoglobin, but rather from the bone marrow due to immature red cell destruction, or to de novo production of stercobilin through an anabolic pathway

A possible degradation or loss of haemoglobin can not be detected in our measurements since the incubation period was limited to 6 hours only Furthermore, it is probable that this loss of haemoglobin occurs in vivo only possibly at the stage where the cells leave the marrow and enter the peripheral circulation. Fessas (6) in a recent study found inclusions of haemoglobin in erythroblasts and erythrocytes of thalassaemia. He concludes that these inclusions represent precipitated haemoglobin which may be eliminated by the red cell.

The per cent utilisation of Fe^{59} for incorporation into the TCA precipitable fraction of the haemolysate is clearly lower in the thalassaemic trait carriers as compared to normals ($p \propto 0.02$) as well as the absolute value of iron (µg) in the same fraction ($p \propto 0.01$) Provided that the decreased iron incorporation into the TCA precipitable fraction of the haemolysate reflects a decrease in haemoglobin synthesis, then our results are in good agreement with

the observations of in vivo studies. Thus the effective erythropoiesis, measured by the red cell Fe^{59} uptake, is lower than normal, rising only to 60–70 / of the administered dose (12) This is also in good agreement with the hypochromia and decreased mean corpuscular haemoglobin constantly observed in thalassaemia trait. ITANO's explanation in this respect is the existence in thalassaemic carriers of an inhibition in haemoglobin A synthesis, which normally is synthesized rapidly (8) The evidence of this inhibition is based on the characteristic microcytosis and the presence of increased amounts of haemoglobin A_2.

There is work in progress in this laboratory at present to correlate the uptake of Fe^{59} by the TCA precipitable fraction of the haemolysates with haemoglobin synthesis.

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Summary

The per cent incorporation of iron, taken up by the trichloracetic acid (TCA) precipitable fraction of haemolysed immature red cell, as well as the amount of iron located in the same fraction were estimated in vitro, using Fe^{59} The per cent incorporation of Fe^{59} was not significantly higher in thalassaemic patients than in normals but it was significantly lower in thalassaemic trait carriers. The amount of iron located in the TCA precipitable fraction of the stroma free haemolysate was considerably higher in thalassaemic patients than in normals. I thalassaemia trait carriers significantly lower values than in normals were observed.

Résumé

Le pourcentage du fer incorporé et la quantité totale de fer sont déterminés dans la fraction d'érythrocytes immatures précipitée par l'acide trichloracétique à l'aide du Fe^{59} Le pourcentage du fer incorporé des malades atteints d'une thalassémie majeure n'est pas plus fort que celui de personnes saines, par contre il est diminué d'une façon significativ dans les cas de thalassémie mineure La quantité totale de fer des fractions examinées est fortement augmentée chez les cas de thalassémie majeure et fortement diminuée chez la thalassémie mineure en comparaison avec des personnes saines.

Zusammenfassung

I der mit Trichloressigsäure fällbaren Fraktion hämolysierter unreifer Erythrozyten wurden die prozentuale Eisenaufnahme und die Gesamteisenmenge mit Hilfe von Fe^{59} in vitro bestimmt. Die prozentuale Eisenaufnahme war bei Patienten mit Thalassaemia major nicht größer als bei Gesunden, dagegen war sie bei Fällen von Thalassaemia minor signifikant erniedrigt. Der Eisengehalt der untersuchten Fraktion

war bei Thalassaemia major beträchtlich größer, bei Thalassaemia minor jedoch signifikant kleiner als bei Gesunden.

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Influence of X Irradiation of the Dog Kidney on Erythropoietin Production

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The kidney has been identified as a primary site of production of an erythropoietic hormone. Jacobson et al. (1) have shown that bilateral nephrectomy renders animals incapable of responding to erythropoietic stimuli such as cobalt, bleeding or depressed oxygen tension. More direct evidence was obtained from studies in which perfusion of the isolated kidney with blood containing cobalt (2) or blood maintained at a low oxygen tension (3, 4) resulted in a significant elevation of erythropoietin titers in the blood perfusate. Mendelsohn et al. (5) have studied the influence of X ray on the excretory function of the kidney; however, very little attention has been given to the effects of ionizing irradiation on erythropoietin production by the kidney. Severe anemia is often seen in association with acute radiation nephritis (6, 7, 8, 9) and the anemia is more severe than would be expected from uremia alone. Since the kidney is known to be a primary site of erythropoietic hormone production, we were interested in learning whether X-irradiation of the kidney would modify the response to an erythropoietic stimulus. Therefore, studies were undertaken utilizing unilaterally nephrectomized dogs, to determine the influence of X ray of the kidney on the response to a cobalt stimulus. Cobalt was chosen as the stimulus because it has been shown to produce a marked increase in kidney erythropoietin production (2). In addition, changes in creatinine and PAH clearances, BUN, hematocrit, blood pressure, baseline plasma erythro-

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Fig. 1 Picture of the dog, with kidney exposed, positioned under the X-ray unit during kidney irradiation.

poietin levels and body weight were studied in dogs after kidney irradiation.

Materials and Methods

Male and female mongrel dogs weighing between 8 and 17 kg were used throughout these studies. On the first day of the experiment and while the dogs were anesthetized with pentobarbital (30 mg/kg) the right kidney was removed and the left kidney exteriorized through retroperitoneal incision, was exposed to 3600 roentgens X-ray. Each dog received 400,000 units procaine penicillin-G and 500 mg streptomycin sulfate subcutaneously as well as 100 ml warm 6% Dextran in 5% dextrose intravenously following surgery. The X-ray was delivered with the use of Westinghouse 240 KV 15 MA X-ray unit with 1 mm copper and 1 mm aluminium filter at 50 cm target to kidney distance. The output was 60 per min. with half value layer of 2 mm of copper over 6 6 cm port. The animals were shielded with two layers of 1/16 thick lead plates with dimensions of 8 8. The mean dosage of radiation to the kidney is estimated to be 3000 rads. Figures 1 and 2 are pictures of the dog in position under the X-ray unit with the kidney exteriorized. The parameters that were measured on the first day of the study and 3 and 6 weeks following kidney irradiation were body weight, blood pressure plasma erythropoietin titers, hematocrit and BUN. Eight dogs were used as controls and 8 dogs were used in the kidney irradiation group. Three and 6 weeks following kidney irradiation, creatinine and PAH clearances were measured while the dogs were anesthetized with pentobarbital. The clearance studies were carried out approximately 1 hour after 400 ml water had been administered via stomach tube. The clearances were carried out using 7.5 mg/kg/min of mannitol infusions as diuretic. The urine was collected through bladder catheter. Fifteen minute periods, with femoral artery blood samples 2 minutes before the endpoint, were used. The PAH and



Fig. [illegible] Picture of exteriorized kidney with lead shielding during kidney irradiation.

creatinine measurements represent an average of 6 periods, a primer dose of 30 micromoles/kg of paraaminohippurate, 100 mg/kg creatinine and 750 mg/kg of mannitol was administered [illegible] minutes prior to initiating the infusion of PAH (1 μM/kg/min) and creatinine (1 mg/kg/min). Creatinine (10) and PAH (11) analyses were carried out on the urine and blood samples at the end of each collection period.

Microhematocrits were determined on the blood sample with heparinized capillary tubes. The arterial pressure was measured via femoral artery needle puncture with the use of a Statham Pressure Transducer and a Grass Polygraph while the dogs were anesthetized with pentobarbital. The blood urea nitrogen was measured by direct nesslerization (1[illegible]). Erythropoietin assay was carried out in the fasted rat according to the procedure of Fried et al. (13). Male rats of the Sprague Dawley strain were used in each erythropoietin assay. Young healthy rats, weighing 165 to 195 grams each, were fasted for 72 hours prior to the first injection of substance being assayed, and maintained in the fasted state throughout the duration of the bioassay, water being permitted ad libitum. Two ml of plasma or the test substances were injected subcutaneously daily for [illegible] days. On the third day a tracer dose of 1 microcurie of Fe^{59} citrate was given intravenously and standards were prepared for later counting. Sixteen hours later 1 ml of blood was obtained by cardiac aspiration and the Fe^{59} incorporation into red cells was calculated according to the following formula:

$$Fe^{59}\ \text{uptake} = \frac{\text{net counts per ml blood} \quad 0.05\ \text{body weight}}{\text{net counts injected}}$$

In these experiments, the blood volume was assumed to be [illegible] of the body weight. A dosage of 25 μM cobaltous chloride per kg was injected subcutaneously as the erythropoietic stimulus in each dog. Approximately 60 ml blood was removed from the femoral artery for erythropoietin assay prior to the cobalt injection and at 1[illegible], 18 and [illegible]4 hours after the injection. Plasma samples were assayed for cobalt by a modification of the technique of Alarcon (14). Each sample contained less than 0.1 micromole cobalt before assay for erythropoietin, which is not sufficient amount to significantly stimulate Fe^{59} incorporation in RBC. Analytical grade cobaltous chloride hexahydrate with maximum impurity for lead of 0.005 [illegible] was dissolved in [illegible] saline before injection. The technique of analysis of variance was used in determining significant differences between experimental groups (1[illegible]). The right kidney which was removed at the beginning of the

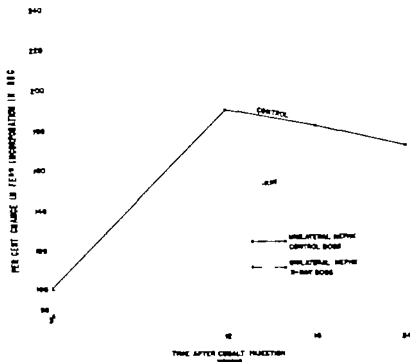


Fig 3 Mean plasma erythropoietin titers following an injection of 250 μM cobalt per kg in dogs 3 weeks after unilateral nephrectomy and sham exposure of the kidney to irradiation or unilateral nephrectomy and contralateral kidney irradiation.

study and left kidney removed at the end of the radiation periods were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin before being studied microscopically

Results

Figure 3 depicts the plasma levels of erythropoietin at 12, 18 and 24 hours after an injection of cobalt in dogs 3 weeks following either kidney irradiation or sham-exposure of the kidney to X ray. A significant increase in erythropoietin levels in plasma was noted 12, 18 and 24 hours following the cobalt stimulus in sham-exposed control dogs 3 weeks after unilateral nephrectomy. Unilaterally nephrectomized dogs whose remaining kidneys were exposed to 3600 r demonstrated a slight but not significant reduction in the erythropoietic response to cobalt. Even though the response was slightly less in the irradiated dogs, plasma erythropoietin titers were significantly elevated above the initial control values. Therefore, it

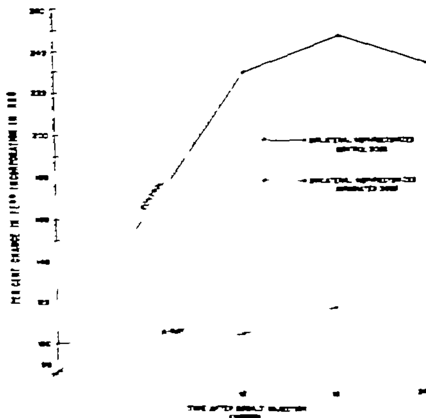


Fig. 4 Mean plasma erythropoietin titers following an injection of cobalt in dogs 6 weeks after unilateral nephrectomy and sham exposure of the kidney or unilateral nephrectomy and contralateral kidney irradiation.

may be concluded that kidney irradiation has not significantly reduced the erythropoietic response to cobalt 3 weeks following kidney irradiation.

Figure 4 demonstrates the plasma levels of erythropoietin during a 24 hour period after an injection of cobalt in dogs 6 weeks following kidney irradiation or sham exposure of the remaining kidney. Erythropoietin levels were significantly less at 12, 18 and 24 hours following the cobalt injection in unilaterally nephrectomized dogs 6 weeks after kidney irradiation when compared with erythropoietin titers in sham controls. The Fe^{59} incorporation values for the 12 hour plasma samples from the control dogs was 229 [illegible] of the normal control value whereas, the mean value in the irradiated dogs was only 105 [illegible] the controls at the 12 hour period. The mean

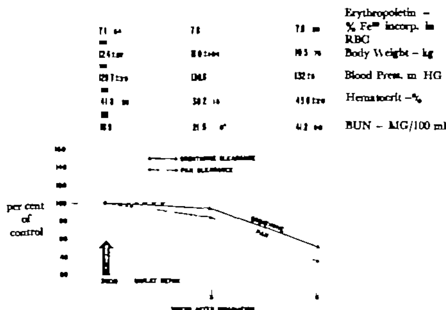


Fig. 5. Effects of X-irradiation of the dog kidney on plasma erythropoietin titers, body weight, blood pressure, hematocrit, BUN and creatinine and PAH clearances. ± is the standard error of the means and indicates significantly different from the 0 day control value at the 5% level.

values in control animals at 18 and 24 hours were 239 / and 222 / of initial controls whereas, the mean values in the X ray group were 118 / and 132 /. The significant reduction in the erythropoietic response to cobalt in dogs 6 weeks following kidney irradiation indicates that X ray to the kidney in a sufficiently large dose, results in a marked suppression of the response of the kidney to an erythropoietic stimulus.

Figure 5 depicts the remaining data obtained from dogs initially and 3 and 6 weeks following kidney irradiation. The control measurements in non-irradiated animals for creatinine and PAH clearances averaged 35.8 ml/min and 323 6 ml/min respectively over the 6 week period. As seen from figure 5 there was a significant depression in the renal clearance values at 6 weeks but the values were not significantly suppressed 3 weeks after kidney irradiation. The creatinine and PAH clearances were lowered to about 96 / and 89 / of controls respectively at 3 weeks post irradiation. However the creatinine clearances were reduced to 50 / and the PAH values to 31 / of control values 6 weeks after kidney irradiation. These findings con-

firm the work of MENDELSOHN et al (5) that kidney irradiation results in a significant suppression of the renal excretory functions. The blood urea nitrogen (fig 5) was found to be significantly elevated at three (21.5 $\pm$ 3 8 mg /) and six (41.2 $\pm$ 9 0 /) weeks post irradiation when compared with the initial 0 day controls (16.9 $\pm$ 1 1 /) This progressive rise in blood urea nitrogen reflects the decrements in creatinine and PAH clearances. The dogs progressively lost weight during the 6 week period following kidney irradiation. The mean hematocrit value was decreased at 3 weeks (36.2 $\pm$ 1 8 /) as compared to 0 day controls (41 8$\pm$0.28 /) but had returned to normal values after 6 weeks (43 6 $\pm$ 2 0 /) The arterial blood pressure and baseline plasma erythropoietin values were not significantly changed during the six weeks following kidney irradiation

It was of interest to us to determine whether stretching of the renal pedicle, exteriorisation of the kidney or positioning of the kidney within the lead shielding during X ray resulted in a change in the response to cobalt or any of the other parameters measured in the irradiated dogs. The response of the sham-exposed control dogs to cobalt shown in figures 3 and 4 indicates that these animals are still capable of responding to a cobalt injection. Table I shows the remaining results obtained in 8 dogs initially and 3 and 6 weeks following unilateral nephrectomy and sham-exposure to the procedure followed in the irradiated dogs. The sham technique (table I) did not significantly change the body weight hematocrit, blood urea nitrogen blood pressure or baseline plasma erythropoietin levels during the 6 weeks following sham kidney exposure. These findings indicate that the changes in renal function observed after kidney irradiation were the result of changes induced by X ray and not the technique employed for exteriorisation of the kidney

Figures 6 and 7 show microscopic sections of the right kidney removed from a dog on the first day of the study and the left kidney from the same dog 6 weeks after irradiating the kidney with 3600 r The irradiated kidneys on gross examination at autopsy were found to be much smaller than the contralateral non-irradiated kidneys. As seen in the microscopic section of the kidney receiving the X ray (fig 7) there was a demonstrable atrophy of the cells of the proximal convoluted tubules, vacuolization of the cells lining the distal tubules, moderate dilatation of Bowman s capsule with a slight amount of protein precipitate in Bowman s space. The proximal tubules showed more marked atrophy than the distal tubules The renal

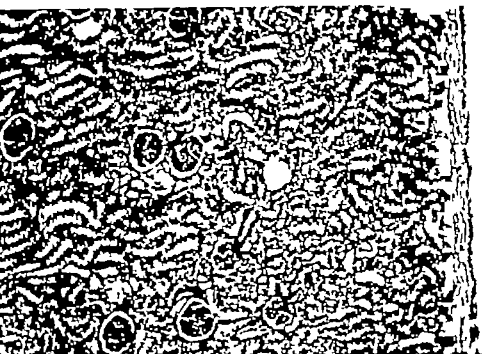


Fig. 6. Microscopic section of normal control right kidney removed from dog prior to irradiation of the contralateral kidney (33 x).

Table I

Influence of unilateral nephrectomy and sham-exposure of the kidney in dogs on body weight, hematocrit, BUN blood pressure and plasma erythropoietin titers.

Time after unilat. nephrec. (weeks)	Body Weight kg	Haematocrit %	BUN mg%	Blood pressure mm Hg	Plasma Erythropoietin Fe^{59} incorp
0	11 1±0.50	36 6±1 40	16.0±0.80	137±4.6	7 3±0 50
3	10 6±1 00	36 6±1.90	23.6±5 10	128±5 1	6 5±0.40
6	10.8±0 80	34 3±0.80	17.6±0.70	133±7 4	7.5±0 04

8 dogs were studied in each group at 0, 3 and 6 weeks following sham exposure;
± Standard error of the mean.

capsule was markedly thickened and fibrotic. Many of the glomeruli showed the appearance of acute membranous glomerulonephritis. There was thickening of the afferent arterioles and intralobular arteries. The glomerular tufts showed a moderate degree of atrophy and Bowman's capsule was thickened with a hyaline material on the inner surface. COGAN et al. (16) have suggested that the glomerulus

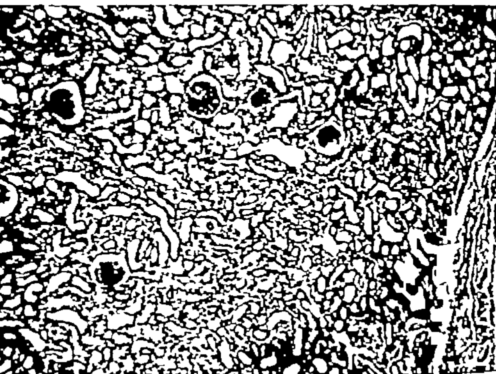


Fig 7 Microscopic section of the left kidney of the dog shown in fig. 1 six weeks after the kidney was irradiated with 3600 (35).

is the primarily affected portion of the kidney and the tubular atrophy is secondary. These findings are similar to those reported by other investigators (5 16 21 22, 23)

Discussion

The present studies demonstrate that irradiation of the kidney prevents the rise in plasma erythropoietin levels which is commonly seen after an injection of cobalt. This effect of X ray to the kidney was developed maximally six weeks following kidney irradiation in the unilaterally nephrectomized dog. The unilaterally nephrectomized dog was chosen in order to prevent the obscuring effects of variable compensatory hypertrophy of the contralateral kidney. This finding may be of significance in clarifying the role of the kidney in erythropoiesis. In this regard it is of interest to note that the erythropoietic response to cobalt was almost completely

abolished six weeks following exposure of the kidney to 3600 r of X ray however the clearances, which are a reflection of the excretory function of the kidney were only reduced by 30–50 / This finding may indicate that erythropoietin production by the kidney is more sensitive to ionizing irradiation than the excretory function.

It is of further interest to speculate from these observations as to the significance of the effects of X irradiation of the kidney on the renal site of production of erythropoietin OSTES (9) has reported that X irradiation of the kidneys in mice results in a hypogranularity of the juxtaglomerular cells. CHAMBERS AND CAMERON (17) have demonstrated a marked resistance of the functioning of renal tubular cells in tissue cultures to radiation. Our finding that the erythropoietic function of the kidney was more markedly depressed by X-ray than the excretory function may be related to a more marked resistance of the renal tubules to radiation than the juxtaglomerular cells. These observations may give further support for the suggestions by other authors (9 18) that the juxtaglomerular cells are the renal site of production of the erythropoietic hormone

It is of interest to ask whether the kidney is only necessary as a source of erythropoietin in panic situations such as after severe hemorrhage marked anemia or hypoxia or does the kidney in addition, elaborate erythropoietin to maintain normal erythropoiesis in providing additional red cells to replenish those lost through normal red cell destruction. HOUCH (19) has shown that there is a significant drop in total circulating red cell volume in bilaterally nephrectomized dogs, maintained via peritoneal dialysis for 111 days, and the anemia had to be treated at intervals of 10 days with blood transfusions. NAETS (20) has also shown that iron turnover rates and radioactive iron incorporation in red cells was significantly depressed as well as a rapid depletion of normoblasts in the bone marrow of dogs after bilateral nephrectomy This author (20) concluded that removal of the kidneys abolishes the capacity of the dog to produce red cells regardless of the severity of the anemic stimulus. A severe normochromic normocytic anemia has also been reported in association with acute radiation nephritis (6) and this anemia is apparently refractory to all forms of therapy except blood transfusion. The anemia is more severe than the associated rise in blood urea nitrogen would warrant (7 8) and therefore must be due to an additional factor other than suppression of the excretory function of the kidney On the other hand, PAGE (21) irradiated

transplanted kidneys in nephrectomized dogs and did not detect anemia, which is regularly observed in human nephritis, in his dogs with Roentgen ray nephritis. It is difficult to draw any conclusions from this work because the blood picture was assessed with hemoglobin determinations alone and no measurements of total circulating red cell volume or bone marrow myelograms were made. Anemia was also reported to be one of the major complications during sixty five prolonged dialyses with the twin coil artificial kidney in human patients with chronic renal failure (22). The hematocrit values in our dogs were depressed after 3 weeks but had returned to normal 6 weeks following kidney irradiation. Apparently there was sufficient functioning renal tissue to maintain normal erythropoiesis after a dosage of 3600 r to the kidney but was insufficient for the kidney to respond to a potent erythropoietic stimulus such as cobalt injection. It seems apparent that the anemia which has been reported to develop after kidney irradiation (6, 9) is due for the most part to suppression in erythropoietin production by the kidney. It is also postulated that the kidney is the source of a single erythropoietic hormone which is necessary for the maintenance of normal erythropoiesis and is also responsible for the polycythemias seen following stressful erythropoietic stimuli such as severe anemia, hypoxia or cobalt. The inability to detect significant plasma levels of erythropoietin in normal humans is probably a reflection of the insensitivity of the assay methods.

Several workers (23, 24, 25) have reported experimental observations on the relationship of hypertension to structural renal damage after X irradiation of the kidneys. Previous work by WILSON et al. (23) has suggested that X irradiation increases the susceptibility of the renal arteries to hypertensive damage. Additional studies by these workers (26) have revealed that the vascular lesions observed in severe hypertension are identical with those found after X irradiation. During the six week period following kidney irradiation in our dogs we did not find a significant change in the mean arterial pressure. It is quite possible that if the animals had been followed for a longer period of time that some evidence of hypertension may have been observed.

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Summary

The kidney exteriorized through a retroperitoneal incision, of unilaterally nephrectomized dogs was exposed to 3600 r in a single exposure from a 240 KV X-ray unit. A marked suppression in the erythropoietic response to an injection of cobalt was observed in the dogs 6 weeks following kidney irradiation. A gradual suppression in the excretory function of the kidney was also seen as indicated by lowered creatinine and PAH clearances at 3 and 6 weeks following kidney irradiation. Blood urea nitrogen values also progressively increased over the 6 week period after irradiation. It may be concluded from these studies that X-irradiation of the kidney in the unilaterally nephrectomized dog results in a significant suppression in erythropoietin production by the kidney and also produces a marked decrease in the excretory function of the kidney. The significance of these findings and their functional interrelationships to erythropoiesis are discussed.

Résumé

Le deuxième rein de chiens nephrectomisés d'un côté est extériorisé par incision rétropéritonéale et exposé à une dose unique de 3600 r d'un tube de 240 KV. La réaction de l'érythropoèse à une injection de cobalt 6 semaines après l'irradiation est nettement diminuée. Parallèlement une diminution progressive des fonctions de l'excrétion mesurée par la clearance de la créatinine et du PAH est observée 3 à 6 semaines après l'irradiation. L'urémie augmente progressivement au cours de ces 6 semaines. On peut conclure que l'irradiation d'un rein de chien déjà nephrectomisé d'un côté produit une diminution de la production de l'érythropoïétine et des fonctions de l'excrétion rénale. Les auteurs discutent la signification de ces données et leur relation avec l'érythropoèse.

Zusammenfassung

Bei einseitig nephrektomierten Hunden wurde die zweite Niere durch eine retroperitoneale Inzision vorgelagert und mit einer 240 KV Röhre einer einzelnen Strahlendosis von 3600 r ausgesetzt. Die Reaktion der Erythropoese auf eine Kobaltinjektion war 6 Wochen nach der Nierenbestrahlung deutlich herabgesetzt. Ferner ergab sich auf Grund einer verminderten Kreatinin- und PAH-Clearance eine zunehmende Herabsetzung der exkretorischen Nierenfunktion 3 und 6 Wochen nach der Nierenbestrahlung. Ebenso stiegen im Laufe dieser 6 Wochen die Harnstoffwerte im Blut progressiv an. Aus diesen Beobachtungen geht hervor daß eine Nierenbestrahlung bei einseitig nephrektomierten Hunden zu einer signifikanten Verminderung der Erythropoetinproduktion und der exkretorischen Funktion der Niere führt. Die Bedeutung dieser Befunde und ihrer Beziehungen zur Erythropoese wird besprochen.

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Further Experience with Streptokinase as Thrombolytic Agent

By A. Amery J Vermylen H. Maes and M. Verstraete

The results obtained in our first series of patients with recent peripheral arterial occlusions treated with streptokinase (SK) have been reported (1) By comparing series of arteriograms taken before, during and after therapy we obtained arteriographic evidence of clot dissolution in 10 out of 22 arterial occlusions in 20 patients. The mean time-interval between start of SK therapy and reappearing of pulsations in these 10 cases was 48 hours. We would wish to stress that even when, as in our series, streptokinase is administrated continuously in relatively high dosis (mean in our series 1 300 000 units/day) it takes an average of two days to obtain thrombolysis. Therefore, we consider treatment with SK to have been sufficient only when streptokinase was given for at least 48 hours, or until clot dissolution occured. We found that 17 out of the 22 occlusions were treated sufficiently long* In 10 out of these 17 occlusions (59 %) arteriographic evidence of thrombolysis was obtained.

We would like to discuss now why in 5 of the 20 patients SK administration was interrupted within 48 hours after the start of therapy In one case, therapy was stopped about half an hour after initiation.

This patient, who received his priming dose in one hour period, developed after approximately twenty minutes very severe reaction. He vomited repeatedly and shivered dramatically his hands, fingers and nose were cold and cyanotic the blood pressure was 100/60 mm Hg the pulse rate 140/min (previously 75/min) Humid râles could be heard over both lungs. As shock seemed imminent, administration of streptokinase was immediately halted and substituted by 25 mg Soludacortine; 10 minutes later the vomiting subsided, the temperature had risen to 39 °C, and the shock symptoms faded. I was decided to discontinue the streptokinase therapy in this patient.

* SK treatment at least during 48 hours or until thrombolysis occured.

Similar but minor reactions were noted in two other patients of our series, when receiving the priming dose but our general impression is that on the whole very high priming doses have been surprisingly well tolerated.

In the four other cases in which the streptokinase administration had to be interrupted within 48 hours, this was due to the death of the patient. Twice, death followed occlusions of the abdominal aorta, once death was due to intracranial hemorrhage and once to shock. It is not easy to state whether an occlusion of the abdominal aorta should be treated with thrombolytic agents. We have treated three such patients with SK, and all three died usually before the streptokinase administration could have had any thrombolytic effect. Nevertheless, aortic occlusions have been removed by streptokinase (2) which is, in the absence of adequate surgical facilities, the only treatment available for this usually fatal condition.

Streptokinase treatment had to be interrupted in one patient, who died from intracranial hemorrhage. Bleeding is a major complication of streptokinase treatment besides ecchymoses at the puncture sites, which occured usually seven of the twenty patients experienced serious bleeding In three cases, a massive haematome developed round the intra arterial catheter left in the femoral artery for the control arteriography in one case, who underwent surgery twelve hours previously important blood loss was observed at the operation site one patient had massive retroperitoneal haematoma following translumbar aortography and another an extensive ecchymosis in the lumbodorsal area, probably related to an intramuscular injection. It should be stressed that in six of the seven cases, bleeding was related to *trauma* which should be anxiously apprehended If all traumata, including intravenous and intramuscular punctures, could be avoided and if suitable administration schedules are used, nearly all hemorrhagic complications would be eliminated, as we observed in our last patient. We believe that, at the present stage of development of thrombolytic therapy in peripheral arterial occlusions, arteriograms are essential and the resulting trauma unfortunately unavoidable Percutaneous femoral arterial catheter introduction is definitely preferred to translumbar needle puncture This catheter which can be left in the artery has several advantages repeated control arteriographies can be made and the frequent blood samples, necessary for following the treatment can be obtained without annoying the patient or provoking

oozing puncture wounds. Because of a fatal intracranial hemorrhage in an hypertensive patient, we have decided not to administrate streptokinase to patients with severe diastolic hypertension.

One patient died in shock during streptokinase therapy This could not be related to blood loss. In our series, a definite but transient fall in blood pressure without evidence of bleeding, occurred in 5 of the 20 patients during streptokinase administration. The mechanism of this hypotension is perhaps related to the transformation of plasma kininogen in plasma kinin during streptokinase treatment (3)

Summary

Streptokinase should be administrated continuously in high amounts during at least two to three days. In these circumstances, there is fair chance of thrombus dissolution.

Résumé

Après une administration continuelle de fortes doses de streptokinase pendant au moins 2 à 3 jours, il y une chance réelle d'une dissolution d'un caillot intravasal.

Zusammenfassung

Bei kontinuierlicher Zufuhr von Streptokinase in hohen Dosen während mindestens 2 bis 3 Tagen besteht grosse Aussicht auf die Auflösung eines Thrombus.

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Serological and Genetical Observations on the Blood Group A_3 B

By C. Levene and I Cohen

The weakly reacting agglutinogen A_3 in the blood of individuals belonging to the groups A and AB was first described by Friedenreich (4) He described the agglutination reaction which is given by anti A from group B persons, and by almost all group 0 serum, as being characterized by small agglutinates in a field of unagglutinated cells (mixed field agglutination)

Gammelgaard (5) also studied the subgroups of A and showed that the subgroups A_3 could be differentiated from what he called A_4 A_5 and A_x by the fact that all A_3 bloods gave only agglutination with anti-A from B persons, but A_4, A_5, and A_x bloods gave only agglutination with 0 sera. The secretion of A substance in the saliva has also been helpful in the distinction of persons with the subgroups A_3 and A_3B In these persons A substance is present in the saliva, in contradistinction to persons of subgroups of A variously designated A_x A_5, A_4, where the saliva does not contain A substance, but contains a substance which inhibits the action of 0 sera against these weak subgroups of A (2) The presence of anti-A_1 in the sera from persons of group A_3 and A_3B has been variously reported by Weiner and Silverman (13) Young and Witebsky (12) Race and Sanger (7) and Dunsford (2) It seems that anti A_1 is only present in the serum of some cases of A_3 and A_3B bloods, but not in all

Our interest in the AB0 subgroups was aroused when during the routine testing of blood groups of antenatal specimens, in the year 1962 two specimens from unrelated women, Mrs. MN and Mrs. ML were found where the apparent group was A_3B, i. e. the red cells when tested with a number of examples of anti A (from

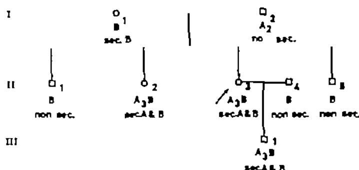


Fig. 1 Pedigree of family N showing ABO blood groups and secretor status.

group B donors) gave a mixed field agglutination pattern The serological reactions of the red cells from both these women were fully investigated, together with the examination of their serum for the presence of anti-A_1 and their saliva was tested for the secretion of A substance. The importance of family studies in these cases has been emphasized by DUNSFORD (3) and the blood groups of the other members of both families were examined. The pedigree of family N is given in fig 1 and that of family L is given in fig 2. From these family studies we found in family N (fig 1) two further examples of the blood group A_3B.

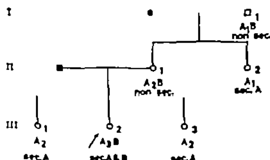


Fig. 2. Pedigree of family L showing ABO blood groups and secretor status.

These two family studies are presented to emphasize the importance of investigating the blood groups of all available members of the family before the final diagnosis of A_3B is confirmed, even though the serological reactions of the individual case may appear of the classical type A_3B.

Materials and Methods

Blood specimens: Clotted blood samples were obtained from all persons investigated. A 2-5 % suspension of the red cells was prepared in normal saline, for testing of the red cell antigens.

Saliva samples were collected and stored according to the method outlined by Sneath et al. (9).

Anti-A and anti-B. These reagents were chosen from persons whose anti-A and anti-B had not been stimulated by the injection of A and B substances. Some of the sera used were from women who had been immunized during the course of pregnancy.

Anti-A_1 was a commercial preparation supplied by the Ortho-Pharmaceutical Corporation, Raritan, N.J.

O serum (anti-A + anti-B) was obtained from a pregnant woman who had high titres of anti-A and anti-B. The serum was absorbed with B cells prior to use to remove all traces of agglutination with B cells.

Anti-H was prepared by saline extraction from the seeds of Ulex Europeus.

Methods. The blood grouping techniques used for the ABO grouping and the titres were performed according to the standard methods described by Race and Sanger (7). Elutions were performed at 56° C according to the method of Landsteiner and Miller (6).

Saliva secretion of ABH substances was tested for by the methods outlined by Boorman and Dodd (1).

Results

Investigation of the red cells

a) Tests with anti A (from group B donors). All four examples of A_3B gave similar reactions with anti-A from group B donors. There was a typical mixed field agglutination picture with many agglutinates dispersed in a field of free cells. The reactions were slow to develop in all cases, and agglutination appeared only after 12–60 seconds in the cases of A_3B, as compared with 3–4 seconds using the same sera with A_1, A_2, A_1B, and A_2B cells.

b) Testing with 0 serum. The serum used had been fully absorbed with B cells to remove all traces of agglutination with B cells. It was noted that the agglutination reaction was quicker to develop (1–2 seconds) than when anti-A from B donors was used. Also the agglutination reaction was complete, and did not give a mixed field agglutination pattern.

c) Tests with anti A_1. The results of tests using anti-A_1 serum are given in tables I and II. It will be seen that groups A_1 and A_1B have given strong reactions with anti-A_1, while A_2, A_2B, and A_3B have given negative results.

d) Tests with anti H. This reagent was prepared from a saline extract of Ulex Europeus. The results are given in tables I and II. The reactions with A_1 and A_1B cells were negative, with A_2 and A_2B cells strong, and weak with the examples of A_3B.

Table I

Results of comparative titres of members of family N using different examples of anti-A and anti-A from 0 serum.

		Control Cells		Donor Cells			
		A_1	A_2	I-2 (A_2)	II-2 (A_3B)	II-3 (A_3B)	III-1 (A_3B)
Serum anti-A	I	1:256	1:128	1:128	1:4	1:4	1:4
	II	1:512	1:256	1:256	1:16	1:16	1:16
	III	1:512	1:256	1:256	1:16	1:16	1:16
Anti-A from 0 serum		1:1024	1:512	1:512	1:128	1:128	1:128
Eluate		1:16	1:32	1:32	1:64	1:32	1:64
Anti-A_1		+++	—	—	—	—	—
Anti-H		—	+++	+++	+	+	+

Table II

Results of comparative titres of members of family L using different examples of anti-A and anti-A from 0 serum.

		Control Cells		Donor Cells					
		A_1	A_2	I-1 (A_1B)	II-1 (A_2B)	II-2 (A_1)	III-1 (A_2)	III-2 (A_3B)	III-3 (A_2)
Serum anti-A	I	1:64	1:16	1:64	1:16	1:32	1:16	1:4	1:16
	II	1:128	1:32	1:128	1:64	1:128	1:32	1:8	1:32
	III	1:256	1:128	1:256	1:128	1:256	1:64	1:16	1:64
Anti-A from 0 serum		1:512	1:256	1:512	1:256	1:512	1:256	1:256	1:256
Eluate		1:8	1:16	1:8	1:4	1:16	1:16	1:32	1:16
Anti-A_1		+++	—	+++	—	+++	—	—	—
Anti-H		—	+++	—	+++	—	+++	+	+++

e) Titration studies The results for family N are given in table I, and for family L are given in table II

1 Titrations using anti A (from B donors) The cells from the propositus in both families, and the other two examples of A_3B in family N show marked reduction in titre in comparison with the control A_1 and A_2 cells. In family L (table II) the titrations were compared with A_1B and A_2B members of the same family

2 Titration using anti A from 0 serum. In family N (table I) the titrations using the red cells of the members of the family with A_3B are slightly reduced as compared with A_1 and A_2 cells. However in family L (table II) the titration score shows no difference from that of A_1 and A_2B members of the same family

f) Elution after absorption using anti A from 0 serum. Although little difference is noted in both families with the strength of the eluate it can be seen from tables I and II that the eluate from the cases of A_3B tends to be slightly stronger than that from the other subgroups of A and AB.

g) Cell typings of other blood group systems The cells were typed for Rh (C, c, D E) and MN blood groups. No mixed field aggluti-

nation was observed using these antibodies and so we can rule out the possibility of their being chimeras.

Investigation of the serum for anti A_1

The serum from the three members of family N (fig 1) with the group A_3B, and the propositus in family L (fig 2) were examined for the presence of anti A_1. Anti A_1 was detected in the serum of all of these individuals.

Saliva secretion

The secretor status of the members of both families are given in figs. 1 and 2. It will be seen that all the examples of group A_3B in both families are secretors.

Discussion

The blood group A_3B is not a homogeneous group and the mere finding of a partial or mixed field agglutination pattern with anti A (group B) cannot be taken as an absolute diagnostic criteria. It is well known that the A gene when combined with the B gene is weaker in its reaction than when it is alone as in group A (11 10). Also it has been shown by DUNSFORD (3) that group A_3B exists in two forms, one a normal type and the other a rarer type which groups like A_3B.

The A_3 gene has been shown by GAMMELGAARD (5) to follow the laws of Mendelian inheritance, so that with all cases of A_3B it is of great importance to conduct family studies. DUNSFORD (3) goes so far as to say that no person should be considered as A_3B unless they have an A_3 parent or offspring.

A number of family studies have been reported of individuals with apparent blood group A_3B, where either one or other of the parents had an A_3 gene, or when separation of the A and B genes occurred in the children, the A gene was expressed as A_3. These family studies were reported by YOUNG AND WITEBSKY (12), SALMON et al. (8) and DUNSFORD (3). In the case described by YOUNG AND WITEBSKY (12) the results were interpreted as an exception to the laws of inheritance, as the mother was A_3B, the father B and the two children had blood groups A_1B and A_3 respectively. In the cases reported by DUNSFORD (3) it was possible to see the combination of A_3 and B genes from the parents producing an A_3B child. The children of the women with apparent blood group A_3B in both

families had children whose group was A_2. These latter cases of A_3B were considered by Dunsford (3) to be due to some form of gene interaction between the A and B genes and not to indicate an exception to the laws of inheritance.

The results of the blood groups of family N (fig 1) are in some ways similar to those of Dunsford (3) in the fact that one parent of the propositus is of group A_2 and the other B The propositus has inherited the A_2 gene from her father and the B gene from her mother and gives a blood group which is classified as A_3B The sister of the propositus also has the blood group A_3B. The propositus (II–3) whose blood group is A_3B, married a man of blood group B and they have only one child whose blood group is also A_3B. Thus it can be seen that in this family the group A_3B has appeared in two generations. The original A gene from the father of the propositus (I–2) was A_2, but when combined with two different group B genes has exhibited serological characters which make it group A_3B. It might be said here, as in the case of Young and Witebsky (12) that a genetic mutation has occurred, and that there has been a change in the A gene. However we do not feel that the available evidence up to date in this family is enough for us *to* say this, as the original A gene was A_2. We feel that here, as in the case of Dunsford (3) this is probably a case of gene interaction where the A gene is weakened by the presence of a B gene, although it would be more convincing if we could see separation of the A gene again from the B gene in this family

The blood groups of our second family L, are given in fig 2. Although they are somewhat incomplete, due to the fact that the grandmother and the father of the propositus were dead, the results show again an association between A_2 and A_3B The propositus in this family groups as A_3B, and her two sisters group as A_2.

It is our feeling from the evidence of our family studies that the examples of A_3B we have found are not true examples, but apparent as in neither family have we been able to find the A_3 gene alone, in the parents or the offspring In both families the resulting A_3B groups have originated from the combination of an A_2 with a B gene. Admittedly in family N (fig 1) the grouping A_3B has appeared in two generations, and could be thought of as an inheritance of A_3 from one generation to another but we feel that more evidence is required from this family before this theory could be substantiated.

Summary

Two family studies have been performed where the propositus in each case was apparently A_3B. In neither case could this grouping be substantiated on the grounds of genetical inheritance of the gene A_3. It is considered in both families that the examples of apparent A_3B are really examples of A_2B, where there is evidence of gene interaction. The reasons for not accepting these cases as true examples of A_3B are discussed.

Résumé

Etude de deux familles suspectes porteurs du groupe sanguin A_3B. Dans les deux familles l'existence de ce groupe sanguin ne pourrait être prouvée. D'après la transmission génétique du gène A_3 les auteurs admettent qu'il [illegible] dans les deux familles avec le groupe apparent A_3B réellement d[illegible] groupe A_2B avec interaction des gènes. Les raisons contraires à l'admission d'un groupe A_3B réel sont discutées.

Zusammenfassung

Es wurden zwei Familien untersucht, in denen ein Träger des Blutgruppenmerkmals A_3B vermutet wurde. In beiden Fällen konnte diese Blutgruppe auf Grund des Erbganges des Gens A_3 nicht bestätigt werden. Es wird angenommen, daß es sich in beiden Familien bei dem scheinbaren Merkmal A_3B in Wirklichkeit um A_2B handelt, wobei eine Wechselwirkung von Genen vorliegt. Die Gründe, die in beiden Fällen gegen die Annahme eines echten Merkmals A_3B sprechen, werden erörtert.

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(Vorstand: Prof. Dr. R. Klima)

Über andauernde Ausschwemmung von Gaucher Zellen ins Blut*

Zytologie und Zytochemie

Von E. Gött und H. Pexa

Gaucher Zellen im peripheren Blut konnten bisher nur vereinzelt nachgewiesen werden. Vielfach wird dies überhaupt bestritten (43, 26, 12). In einem eigenen Fall konnten wir seit mehr als 4 Jahren in zunehmendem Maße Gaucher Zellen im Blut nachweisen und hatten damit die Möglichkeit, ihre morphologischen, biologischen und zytochemischen Besonderheiten zu studieren. Ihr Nachweis war möglich durch die Anwendung des Leukozytenkonzentrates nach Klima et al. Die Zellen waren identisch mit den im Sternalpunktat, in der Leberbiopsie und im Milztupfpräparat vorhandenen Elementen.

Methodik

I. Technik des Leukozytenkonzentrates n. Klima et al. (23).
II. Eine Modifikation, das L.E. Zentrifugat, wurde zum Nachweis von Phagozytosen verwendet. Methodik s. bei Gött (18, 19).
III. Beobachtung der lebenden Gaucher-Zellen im Anoptralverfahren; Reichert Zetopan, Universalkondensor Polyphos, Anoptralkontrast-Ölimmersions-Objektiv 100 : 1.
IV. Verwendete Färbemethoden bzw. zytochemische Reaktionen
1. May-Grünwald-Giemsa (M.G.G.)
2. Nilblausulfat: Methode nach Gomori (20).
3. Perjodsäure-Schiff-Methode (PAS); Methode von Hotchkiss, ausführlich besprochen bei Mitus et al. (33).
4. Sudan Schwarz B; Methode nach Lison, zit. bei Undritz (45).
5. Eisenfärbung; Methode nach Bilger und Tetzner (8).
6. Saure Phosphatase: Methodik bei Rozenszajn and Efrati (41).
7. Alkalische Phosphatase; Methode nach Kaplow (21).
8. Adenosintriphosphatase (ATPase); Bleimethode nach Wachstein and Meisel (47). Für hämatologische Zwecke verwenden wir die Fixierung mit Formol-Chloralhydrat bei +4 °C nach Baker and Firminger (5), wie sie Lennert und Remmele (25) anführen.

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9. Unspezifische Esterase mit alpha-Naphthylacetat als Substrat; Methode nach LÖFFLER (27, 28)
10. Aryl-Sulfatase: Methode nach AUSTIN AND BISCHEL (4)

Kasuistik

Mutter (60 J.) und Schwester (41 J.) leiden ebenfalls an M. Gaucher. Mit 10 J. Tonsillectomie. 1941 Cholecystitis und Cholelithiasis mit Icterus. Dabei wurde eine vergrößerte Milz festgestellt. Im Sternalpunktat waren Gaucher-Zellen nachweisbar. 1944 wurde eine 2.900 g schwere Milz exstirpiert (Krankengeschichte durch Kriegseinwirkung verlorengegangen). Die noch vorhandenen Milztupfpräparate enthalten vorwiegend typische Gaucher-Zellen. 1957 Zahnextraktion ohne wesentliche Nachblutung. März 1959 erstmals Schmerzen im Bereich der Sacroiliacalgelenke. Knochenbiopsie: Die Markräume von einem reticulär-fibrösen Gewebe durchsetzt mit schütteren, blutbildenden Zellinseln. Örtlich auch Gruppen etwas größerer, einkerniger Zellen mit großen, mehr schmutzig-kantigen Plasmaleibern, welche wahrscheinlich Gaucher-Zellen entsprechen. Diagnose: Speicherzell-Myelofibrose (Morbus Gaucher). April 1959 Ischiasbeschwerden rechts, normaler Röntgenbefund des Skeletts. März 1960 Laparoskopie: Beide Leberlappen stark vergrößert, sehr plump, der rechte über handbreit unter den Rippenbogen reichend, blaßbräunlich, mit deutlichen, unscharf begrenzten weißlichen Infiltraten. Biopsie aus dem rechten Lappen: Die mächtig ausgeweiteten Radiärkapillaren sind vollgestopft mit großen, schaumigen Speicherzellen. Die Leberzellbalken dadurch stärker atrophisch, teilweise völlig untergegangen. Auch die Periportalfelder erscheinen vielfach durch die Speicherzellnester komprimiert. Diagnose: Hochgradige retikuloendotheliale Speicherzellwucherung (M. Gaucher). (Die histologischen Befunde verdanken wir dem Vorstand des Patholog. Institutes des Kaiserin-Elisabeth-Spitals, Herrn Prof. Dr. G. HARTMANN.) März 1961 erstmalig ausgedehntere Knochendestruktionsherde im Beckenbereich. Röntgentherapie. Juni 1962 weitere Destruktionsherde im Schenkelkopf links. Oktober 1962 Sklerosierung im Bereich des ganzen Beckens und weitgehende Rückbildung des Destruktionsprozesses links, neuer Herd im Femurschaft links. Februar 1963 unveränderter Röntgenbefund. (Die Röntgenbefunde verdanken wir dem Vorstand des Röntgeninstitutes des Kaiserin-Elisabeth-Spitals, Herrn Doz. Dr. J. BARTSCH.)

Status und Befunde vom Februar 1963: Allgemeinzustand gut, nur geringe, schmerzbedingte Gehbehinderung in den Hüftgelenken. Deutliche bräunliche Pigmentierung der gesamten Körperoberfläche, besonders jedoch der belichteten Körperstellen. Leber 3 Querfingerbreiten unter den Nabel reichend. Ery 3.370.000, Hb 73% /11,5 g%, FI 1,09, Retikulozyten 11‰, Leuko. 7.800, Differentialblutbild im wesentlichen o. B. Im Leukozytenkonzentrat finden sich jedoch neben den normalen Blutzellen seit einigen Jahren konstant in geringer Zahl auch Myelozyten und Promyelozyten, ganz vereinzelt auch Myeloblasten sowie ziemlich häufig Megakaryozytenkerne. Die Erythrozyten zeigen eine ausgeprägte Anisozytose und Polychromasie, in geringer Zahl sind auch Normoblasten auffindbar. Die Thrombozytenzahl schwankt zwischen 42.000 und 155.000. Index der alkalischen Leukozytenphosphatase 69. Senkung (Westergren) 25/52 mm. Koagulation (Weltmann) 1–6 Röhrchen. Serumbilirubin (direkt) 1,08 mg% bzw. (indirekt) 0,58 mg%. Thymoltest 8 E. Zinksulfattest 8 E. Bromthaleintest (60 Min.-Wert) 14 mg% (deutlich pathologisch). Gesamteiweiß 6,43 g%. Albumine 48, Globuline alpha₁ 6,4, alpha₂ 8, beta 12,8, gamma 24,8%. Saure Serumphosphatase 18,72 KAE. Alkalische Serumphosphatase 14,40 KAE. GOT 30 WE, GPT 20 WE, LDH 430 WE, SDH 0,55 E. Serum-Harnsäure 3,54 mg%. Cholesterin (gesamt) 179 mg%, Cholesterin-Ester 105 mg% = 59%, freies Cholesterin 74 mg% = 41%. Serum-Eisen 135 gamma %, latente Eisenbindungskapazität 51 gamma %, totale Eisenbindungskapazität 186 gamma %. Blutzucker 122 mg%. Gesamtlipoide

600 mg%. Serum-Calcium 10,98 mg%. Serum-Phosphor 2,94 mg% Blockingtest negativ Direkter Coombstest negativ Stärke-Gel-Elektrophorese Haptoglobintyp 2 1 Blutgruppe A_1 Rhesus neg Rumpel-Leede scher Stauungsversuch deutlich positiv Gerinnungszeit 15 Min. (verlängert). Nachblutungszeit 3 Min. Harnbefund im wesentlichen im Bereich der Norm. Ekg und Blutdruck o. B.

Bisheriger Gesamtablauf der Krankheit Die ursprüngliche hämorrhagische Diathese hat sich nach der Splenektomie gebessert. 15 Jahre nachher erste Knochenbeschwerden, jedoch anscheinend nur ganz allmählich progressiv Knochenveränderungen. Im letzten halben Jahr war der Zustand bei geringen Beschwerden stationär Bis zum Oktober 1963 keine wesentliche Änderung

Ergebnisse

Die Gaucher Zellen, die im üblichen Blutausstrich nicht gefunden wurden, waren mit Hilfe des Leukozytenkonzentrates andauernd und meist ohne besondere Mühe zu finden pro Objektträgerausstrich konnten maximal bis über 100 Zellen gezählt werden.

I Färbung nach M G G. Man kann deutlich junge und voll entwickelte Gaucher Zellen unterscheiden sowie nach der Art ihrer Speicherung fibrilläre und amorph-körnige. Die *jungen Zellen* haben die Größe etwa eines Monozyten (Abb 1) das Plasma ist basophil, verhältnismäß g dunkel und zeigt meist keine wesentliche Speicherung Der Kern ist in der Regel noch relativ groß in der noch lokkeren Struktur können 2 bis 3 Nukleolen erkennbar sein. Zwei- und auch dreikernige Zellen kommen vereinzelt vor Mit zunehmender Reifung und Speicherung wird das Zytoplasma breiter seine Basophilie nimmt ab und die charakteristische streifig-fibrilläre Plasmastruktur tritt auf. Der Kern wird kleiner und pyknotischer und rückt immer mehr an die Peripherie.

Die voll *entwickelte Gaucher Zelle* (Abb. 2) erreicht schließlich auch im Blut eine Größe bis über 50μ. Zwischen den Fibrillen finden sich wenige bis zahllose, körnige Elemente bis zur Größe von etwa 1μ, die sich basophil anfärben es handelt sich dabei wohl um Organellen Die Zellbegrenzung ist unregelmäßig und unscharf, der Kern mehr oder weniger pyknotisch, nur vereinzelt sind auch noch Nukleolen erkennbar Die einkernigen Zellen überwiegen bei weitem. Diese von uns als *Typ I* bezeichnete Form der Gaucher Zellen überwiegt bei unserer Patientin im Blut ebenso wie in den Geweben. Daneben finden wir wenn auch seltener einen zweiten Typ der Gaucher Zellen (Abb 3 und 4) Die Zellen des *Typ II* haben eine körnige bis amorphe Zytoplasmastruktur mit verschieden stark ausgebildeter Basophilie Ihre Kerne sind meist starker

pyknotisch bzw. degenerativ verändert. Ganz vereinzelt finden sich auch kernlose solche Plasmakörper.

2. L.E. Zentrifugat. Nachdem in der Literatur einige Hinweise auf Zytophagie durch Gaucher Zellen vorhanden sind (s. später) haben wir versucht, diese in dem dafür besonders geeigneten L.E. Zentrifugat nachzuweisen. Wir konnten dabei in keinem Fall eine Phagozytosetätigkeit beobachten. Auch Phagozytosen durch andere Zellen waren nicht auffindbar.

3. Anoptralbeobachtungen. Der Vorteil dieser Art der Beobachtung ist die eindrucksvolle, nahezu plastische Darstellung der vielfältigen Strukturen der lebenden Zelle sowie die Möglichkeit, Lebensäußerungen zu beobachten (Abb. 5, 6, 7). Der Durchmesser der Gaucher Zellen schwankt zwischen 15 und 85μ. Es ist anzunehmen, daß die extrem großen Zellen die Kapillaren nicht passieren können und auf dem Wege über arteriovenöse Anastomosen in das venöse Blut gelangen. Das Zytoplasma läßt deutlich ein Endoplasma und ein Ektoplasma unterscheiden.

Das *Endoplasma* zeigt hier besonders plastisch die Fibrillenstruktur. Die Fibrillen liegen zum Teil einzeln, häufig jedoch in gröberen Bündeln und Balken vereinigt und bilden ein ungeordnetes Netzwerk. Dazwischen finden sich reichlich Mitochondrien und andere Organellen als stark lichtbrechende punktförmige Gebilde bis zur Maximalgröße von etwa 1μ.

Der Zellrand wird vom homogenen *Ektoplasma* gebildet. Es zeigt nur sehr wenig lichtbrechende Strukturen. Seine Begrenzung ist bei der lebenden Zelle wohl scharf, jedoch ausgesprochen unregelmäßig infolge zahlreicher Ausstülpungen, die sich dauernd umbilden, ohne daß es dabei jedoch zu einer Fortbewegung der Zelle kommt. Die Ausstülpungen können sich abschnüren und sind dann als Kugeln in der Umgebung der Zelle anzutreffen. Einmal konnte die Ausbildung eines Schlauches vom Endoplasma durch das Ektoplasma hindurch beobachtet werden (Abb. 6). Das peri-

Abb. 1. Junge, basophile Gaucher Zelle; M. G. G.
Abb. 2. Al... zweikernige Gaucher Zelle mit fibrillärer Struktur (Typ I). M.G.G.
Abb. 3 und 4. Gaucher Zellen mit amorph-körniger Struktur (Typ II). M.G.G.
Abb. 5. Kleine Gaucher Zelle mit fibrillärer Struktur (Typ I) neben segm. Granulozyten. Anoptral.
Abb. 6. Große Gaucher-Zelle mit fibrillärer Struktur (Typ I), zwei Kernen, lebhaftem Ektoplasma und Schlauchbildung unten. Anoptral.
Abb. 7. Gaucher-Zelle mit inaktivem Ektoplasma (tote Zelle?). Anoptral.
Alle Abbildungen haben die Endvergrößerung 1 : 1600.

1



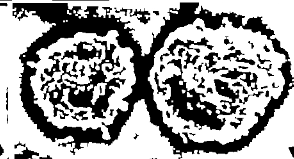
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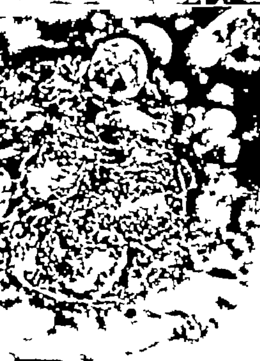


3











obere Ende dieses Schlauches trat dann aus dem Ektoplasma aus und löste sich schließlich ab. Nur ganz vereinzelt konnten Zellen mit einem anscheinend völlig inaktiven Ektoplasma gefunden werden (tote Zelle?) (Abb. 7)

Die *Zellkerne* liegen im Endoplasma, erreichen eine Größe von 7 bis 15 μ und enthalten ein bis zwei, vereinzelt auch drei, bis zu 5μ große Nukleolen.

In einzelnen Zellen finden sich im Endoplasma neben den *Fibrillen wenige bis zahlreiche, nicht lichtbrechende Vakuolen von* etwa 1 bis 3μ Durchmesser (Abb. 9). Die Zellen erhalten damit ein schaumiges Aussehen (*«Schaumzellen»*).

Vereinzelte Zellen haben die bereits bei der M. G. G. Färbung beschriebene Plasmastruktur des *Typ II* (Abb. 8), diese körnig-amorphen Strukturen sind etwa ebenso stark lichtbrechend wie die Fibrillen des Typ I.

Neben diesen Zellformen finden wir manchmal Elemente in einer Größe bis zu 80μ ohne erkennbaren Kern, darin sind, offenbar aus den Fibrillen entstandene, stark lichtbrechende, runde und ovale Ringe bzw. Schläuche und Kugeln und auch Mitochondrien enthalten (Abb. 10). Es dürfte sich dabei um *Umbau- bzw. Abbauformen* der Gaucher Zellen handeln. Außen herum ist oft noch ein verschieden breiter Saum anzutreffen, wohl als Rest des Ektoplasmas.

Daneben sieht man schließlich ganz vereinzelt noch ebenso große, unregelmäßig geformte aber schon nahezu strukturlose Elemente, die die Endphase des Abbaues darstellen (Abb. 11).

4. Nilblausulfat-Färbung. Kern und Plasma sind deutlich blau gefärbt, wobei hier die fibrilläre Plasmastruktur gut erkennbar ist.

5. PAS-Färbung. Während die Granulozyten eine intensivrote Färbung ihres Plasmas zeigen, ist das Plasma der Gaucher-Zellen nur ganz schwach hellrosa angefärbt und eine fibrilläre Struktur nur an einzelnen Stellen erkennbar. Genau das gleiche Aussehen bieten die Gaucher Zellen nach Vorbehandlung mit Diastase bzw. Speichel, während die Granulozyten nach Einwirkung dieser Fermente vollkommen entfärbt sind.

6. Sudanschwarz B-Färbung. Das dunkelgraue Plasma läßt wieder eine deutliche Fibrillenstruktur erkennen, die Kerne sind infolge der Gegenfärbung mit Giemsa rötlich gefärbt.

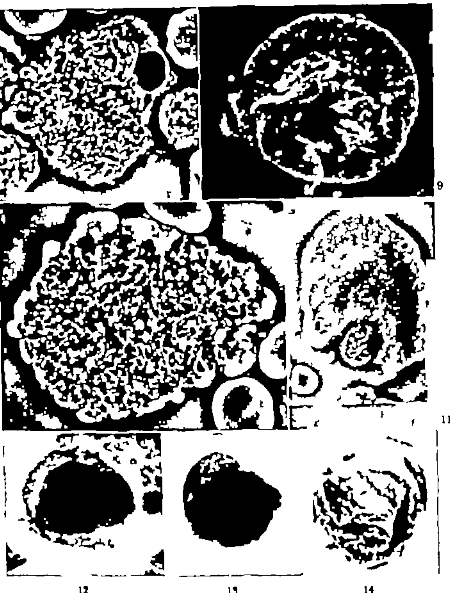


Abb. 8 Amorph-körnige Gaucher-Zelle (Typ II) mit einer großen Vakuole neben dem Kern; Anoptral.

Abb. 9. Fibrilläre Gaucher-Zelle (Typ I) mit Vakuolen («Schaumzelle»); Anoptral.

Abb. 10. Umbauform der Gaucher-Zellen. Anoptral.

Abb. 11 Abbauform der Gaucher-Zellen; Anoptral.

Abb. 12 und 13. Darstellung der sauren Phosphatase mit Gegenfärbung des Kernes.

Abb. 14 Saure Phosphatase ohne Gegenfärbung.

Abb. 11 hat eine Endvergrößerung von 1 : 800, alle übrigen Abb. eine solche von 1 : 1600.

7 *Eisenfärbung* Sämtliche Gaucher Zellen ließen eine Anfärbung vermissen. Im ungefärbten Plasma war eine fibrilläre Struktur zu erkennen.

8. *Saure Phosphatase* Bei allen Zellen konnte eine Aktivität der sauren Phosphatase in Form von braunschwarzen Niederschlägen von Bleisulfid nachgewiesen werden. Sie liegen manchmal zentral und überdecken zum Teil den Kern (Abb. 12) manchmal ist das ganze Plasma ausgefüllt (Abb 13) mitunter tritt die fibrilläre Struktur hervor (Abb 14)

9 *Alkalische Phosphatase* Eine alkalische Phosphataseaktivität ist nicht nachweisbar

10 *Adenosintriphosphatase* Das Plasma der Gaucher Zellen färbt sich schwach bis intensiv schmutzig bräunlich an. Eine fibrilläre Struktur ist mäßig gut erkennbar

11 *Unspezifische Esterase* Das Zytoplasma färbt sich mäßig bis intensiv rotbraun an, zum Teil in körniger Form keine sicher nachweisbare fibrilläre Struktur

12 *Arylsulfatase* Im Plasma der Gaucher Zellen finden sich braune, amorphe Massen sowie manchmal auch eine braune fibrilläre Struktur

Diskussion

Die Gaucher Zellen, die ansonsten in den verschiedensten Geweben gefunden werden (7 9 24 42) konnten wir bei unserem Fall erstmalig regelmaßig und andauernd auch im Blut nachweisen. Im Schrifttum wird ein solcher Befund nur ganz selten angeführt und auch bei wiederholten Untersuchungen wurden nur einzelne Zellen registriert (15 1 44 41 49) Das Vorkommen von typischen Gaucher Zellen im peripheren Blut unserer Patientin erwähnte RIEDER (37) bereits 1959 im Rahmen einer Diskussionsbesprechung

Wie aus den Ergebnissen hervorgeht lassen sich auf Grund der Zytomorphologie und Zytochemie folgende Aussagen machen

1 Wir fanden im Blut neben ausgereiften auch jüngere Gaucher Zellen, wie sie ansonsten nur im Knochenmark beschrieben wurden (39 43 Vakuolenbildungen bzw «Schaumzellen die in Milz und Knochenmark beschrieben wurden (17 41) sahen wir im Blut nur ganz vereinzelt. In den Vakuolen fanden wir keine lichtbrechenden Substanzen, hingegen jedoch in den daneben vorhandenen Fibrillen bzw Granula, die den Lipoproteinkomplex enthalten (Abb. 9) Sowohl PICK (36) als auch BLOCK AND JACOBSON

(9) sehen nur in der fibrillären Struktur das Charakteristikum der Gaucher Zellen. Wir möchten daher meinen, daß der Terminus *Schaumzelle* besser für die spezifischen Zellen bei M. Niemann-Pick und M. Hand-Schüller-Christian reserviert bleiben sollte, bei denen die Speicherung in Tropfenform erfolgt.

2. Auf Grund ihrer verschiedenen Endoplasmastruktur lassen sich *2 verschiedene Typen* von Gaucher Zellen unterscheiden

Typ I weist die allgemein als charakteristisch angesehene Morphologie auf die meisten Autoren bezeichnen diese Struktur als fibrillär (2 9 14 17 32, 34, 48) einzelne auch als knittrig bzw faltig (22, 39 49) und nur wenige sprechen von sogenannten «Schaumzellen» (s.o.) Dieses für die Gaucher Zellen spezifische Charakteristikum kommt bei den meisten Färbemethoden zur Darstellung ebenso wie auch im Phasenkontrast bzw Anaptrolverfahren, im polarisierten Licht und schließlich auch im Elektronenmikroskop.

Phasenoptisch stimmen unsere Beobachtungen mit denen von DeMarsh and Kautz (14) Rohr (38) und Rozenszajn and Efrati (41) überein, ebenso wie mit den polarisationsoptischen Untersuchungen von Anitschkow (2) und Mirmahl und Kübler (32).

Elektronenmikroskopisch stellen sich die Fibrillen als breite Bänder mit irregulärer Begrenzung dar die des öftern verzweigt und von einer Membrane eingehüllt sind. Sie enthalten dichtwandige tubuläre Strukturen, die offenbar den Lipoproteinkomplex darstellen (14 16). Weiters finden sich Anhaltspunkte für die Annahme, daß die Speicherung innerhalb der Mitochondrien beginnt (16, 40) Die Tubuluselemente in den Fibrillen stellen möglicherweise veränderte Mitochondrienreste dar was eine Beteiligung der Mitochondrien am Aufbau der spezifischen Fibrillenstruktur wahrscheinlich macht. Ähnliche Strukturen wurden in Leberzellen als Lysosome identifiziert (3) d.h. als Plasmabestandteile, die eine endogene Lyse der Mitochondrien bewirken können und die vermutlich durch ihre hydrolytische Aktion Produkte erzeugen könnten, die für eine Neuorientierung der Zellfunktion nötig sind. Vielleicht führen derartige Vorgänge in den Zellen des RHS zu einer Funktionsänderung im Sinne einer übermäßigen Aufnahme und Speicherung der Cerebroside oder einer exzessiven Cerebrosidsynthese zur Diskussion steht auch noch ein verminderter intrazellulärer Lipoidabbau (24) Bemerkenswert ist, daß die Aktivität der sauren Phosphatase sowohl in den Lysosomen als auch im Plasma der Gaucher-Zellen deutlich nachweisbar ist.

Typ II zeichnet sich durch eine körnige bis amorphe Plasmastruktur aus und wurde vereinzelt beschrieben von Allemann (1) Födisch (17) Lüdin (30) Mirmahl und Kübler (32) Rohr (39) und Rozenszajn and Efrati (41) Typ II entsteht wahrscheinlich aus Typ I durch Umbau des Cerebrosides in seine kristalloide Form mit Verlust der Fibrillenstruktur

In den eigenen Präparaten fand sich dann noch eine weitere Form des Umbaues mit Ausbildung einer ring kugel- und

schlauchförmigen Struktur (Abb. 10) die durch Abbau in ein homogenes Gebilde übergeht (Abb 11) Diese beiden Formen wur den bisher in der Literatur noch nicht erwähnt. In manchen Zellen konnten wir den Übergang von Zellen des Typ I in solche vom Typ II ebenso wie den Umbau in die beiden zuletzt genannten Formen deutlich erkennen.

3 Im *Ausstrichverfahren* trat besonders eine ausgeprägte Ober flächenaktivität des Ektoplasmas hervor auf die von einzelnen Autoren auch bei den Zellen aus dem Gewebe hingewiesen wird (14 38, 41) Darüber hinaus fanden wir noch die beschriebene aus dem Endoplasma hervorgehende Schlauchbildung mit Ausstülpung und Ablösung

4 Im Gegensatz zu einzelnen Mitteilungen (10 13, 29) konnten wir mit dem dafür besonders geeigneten *L.E. Zentrifugat* bei wiederholten Untersuchungen niemals Phagozytosephaenomene nachweisen.

5 Durch die Anfärbung mit *Nilblausulfat* unterscheiden sich die Gaucher Zellen von den spezifischen Zellen bei M.Niemann-Pick, die ungefärbt bleiben (24 41)

6. Mit der *PAS-Färbung* lassen sich die Glykolipoide zufolge ihrer Kohlehydratkomponente darstellen. Reines Kerasin gibt eine positive PAS-Reaktion (14) und es wird auch die Reaktion in den Gaucher Zellen aus dem Gewebe allgemein als positiv angegeben, jedoch kann ihr Ausmaß je nach der Art der Fixierung variieren. Die Zellen aus dem Blut gaben dieselbe positive Reaktion, jedoch durchwegs nur in geringer Stärke. Über die PAS-Färbung liegen bereits eingehende Untersuchungen vor (6, 16, 31 41)

7 Mit *Sudanschwarz B* färben sich nach Undritz (45) die Lipoide, nach Pearse (35) neben dem Neutralfett besonders die Phospholipoide und in geringem Maße die Cerebroside. Die Gaucher Zellen aus dem Blut färben sich dunkelgrau an ebenso wie die Zellen aus dem Gewebe bei Lüdin (30) Kübler (24) Maloney and Cumings (31) und Rozenszajn and Efrati (41)

8 Die *Eisen*reaktion war bei unseren Untersuchungen durchwegs negativ bei Gewebeuntersuchungen wurden sowohl positive als auch negative intracelluläre Reaktionen gefunden (9 29 17 31) Die positiven Befunde könnten durch den Einfluß hämolytischer Prozesse bzw einer hämorrhagischen Diathese mit Ablagerungen des Hämoglobineisens in Gaucher Zellen zustande kommen.

9 Fermentreaktionen wurden bei M Gaucher bisher nur selten durchgeführt. Die *unspezifische Esterase*reaktion war in unseren Zellen aus dem Blut ebenso wie bei Fisher and Reidbord (16) in den Zellen aus dem Gewebe intensiv positiv Über das Verhalten der *Adenosintriphosphatase* – bzw *Arylsulfatase*aktivität in den Gaucher Zellen fanden wir bisher keine Angaben. Die Reaktion für die ATPase war bei uns schwach bis intensiv positiv und für die Arylsulfatase deutlich positiv

Die Aktivität der *sauren Phosphatase* wurde in den Zellen aus dem Gewebe mehrfach untersucht (11 16) Rozenszajn and Efrati (41) die sich ausführlich damit beschäftigten fanden noch in 8 Monate alten Präparaten bei 60 / der Gaucher Zellen eine Aktivität. Ob die meist auch sehr starke Aktivität der sauren Serumphosphatase aus den Gaucher Zellen stammt, konnte bis jetzt noch nicht geklärt werden. Auf die möglichen Beziehungen zwischen den Mitochondrien, den Fibrillen in den Gaucher Zellen sowie den Lysosomen, denen allen eine deutliche saure Phosphatase aktivität eigen ist, wurde bereits eingegangen.

Die *alkalische Phosphatase*aktivität fanden wir in den Gaucher Zellen des Blutes ebenso wie Fisher and Reidbord (16) im Gewebe negativ Bei diesen Autoren sowie in der Arbeit von Lüdin (30) finden sich weitere Angaben über histochemische Untersuchungen.

Zusammenfassung

Mit dem Leukocytenkonzentrat waren erstmals in einem Fall ständig ausgereifte und auch junge Gaucher-Zellen (G.Z.) im Blut nachweisbar; die meisten hatten fibrilläre Struktur (Typ I) einige amorph-körnige (Typ II) vereinzelte waren vakuolisiert (»Schaumzellen«) Eigenartige Umbauformen und Abbaukörper werden beschrieben. Die G.Z. hatten eine aktive Oberfläche, doch fehlte Phagozytose. Nilblausulfat, Sudanschwarz B und die PAS-Färbung waren positiv die Eisenreaktion negativ An Fermenten fanden sich reichlich saure Phosphatase, deutlich unspezifische Esterase und Arylsulfatase sowie wechselnd ATPase, jedoch keine alkalische Phosphatase.

Summary

For the first time it has been possible by leukocytic concentration to demonstrate regularly in one case the presence of both mature and juvenile Gaucher cells in the blood. Most of them presented fibrillary structure (Type I) while some were amorphous-granular (Type II) and few vacuolar ("foam-cells") Peculiar transitional forms and disintegration bodies are described. The surface of these cells was active, but there was no phagocytosis; the Nile blue sulphate, Sudan black B and PAS staining reactions were positive and iron negative. Enzymes included plentiful acid phosphatase, clearly non-specific esterase and arylsulphatase and occasionally ATPase, but no alkaline phosphatase.

Résumé

Pour la première fois les auteurs ont pu démontrer par la concentration des leucocytes dans un seul cas la présence continuelle de cellules Gaucher (CG) adultes et parfois jeunes dans le sang périphérique. La plupart présentait une structure fibrillaire (type I) quelques-unes étaient amorphe-granulées (type II) ou rarement vacuolées (type de Schaumzellen) Quelques formes de transition et produits de déstruction sont décrits. Les CG ont une surface active alors que la phagocytose fait défaut, les colorations au bleu de nil, noir sudan B et PAS étaient positives, la réaction au fer négative Les CG renferment beaucoup de phosphatase acide, assez d'estérase non spécifique et d'arylsulfatase ainsi que des quantités variables d'ATPase, mais pas de phosphatase alcaline.

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Varia

Deutsche Hämatologische Gesellschaft

Die Jahrestagung 1964 der Deutschen Hämatologischen Gesellschaft findet am 9. und 10. Oktober in Tübingen statt. Hauptverhandlungsthema wird sein «Das Knochenmark und seine Erkrankungen» mit den Gesichtspunkten Cytologie und *Histologie des Knochenmarks, Knochenmarksinsuffizienz* (Agranulocytose, aplastische Anämie) myeloproliferative Erkrankungen, immunologische Erkrankungen, Knochenmarktransfusion. Anfragen und Vortragsanmeldungen sind zu richten an den Vorsitzenden, Herrn Prof. Dr. K. Betke, Tübingen, Univ. Kinderklinik. Anmeldeschluß für Vorträge ist der 30. Juni 1964.

Libri

Heparin and Thromboplastin with Survey on von Willebrand's Disease Hgb. von *I. S. Wright, F. Koller* und *E. Beck* (Schattauer Verlag, Stuttgart 1963). XII 324 Seiten, 120 Abbildungen, 58 Tabellen. Preis Leinen DM 59.– kart. Broschur DM 55.

Der Band enthält alle Vorträge und Diskussionsvoten einer Konferenz, die vom 15. bis 18. Juli 1962 in Stockholm unter dem Patronat des «International Committee on Blood Clotting Factors» 72 Fachleute aus der ganzen Welt vereinigte. An der Tagung sprachen Forscher von internationalem Rang über die aktuellsten Probleme der Blutgerinnung. Ein einleitendes Referat orientiert über den heutigen Stand der Kenntnisse im ganzen Fachgebiet. Dann kommen Vorträge über Heparin, seine chemische Zusammensetzung, seine Wirkung auf den Gerinnungsvorgang und seine therapeutische Verwendung, gefolgt von einer Übersicht über Physiologie und Pathophysiologie der Mastzellen. Ein weiterer Teil ist der von Willebrand'schen Krankheit gewidmet, die heute im wesentlichen als Coagulopathie und nicht als Thrombopathie aufzufassen ist. Das nächste Kapitel handelt von einer Phase der Gerinnung, die besonderes Interesse beansprucht, von der Bedeutung und Wirkungsweise des Thromboplastins, mit Vorträgen über Thromboplastin im Extrinsic System und Intrinsic Thromboplastin. Am Schluß stehen Berichte internationaler Fachgremien über die Kontrolle der Anticoagulantientherapie, über Hämostase und neue Gerinnungsfaktoren. Allen Vorträgen schließt sich unmittelbar eine meist sehr aufschlußreiche Diskussion an, die den Wert des Buches noch erhöht. Der Band der Stockholmer Tagung vermittelt auch dem nicht auf dem Gebiet der Gerinnungsphysiologie tätigen Hämatologen eine ausgezeichnete und willkommene Zusammenfassung der neuesten Kenntnisse über den Gerinnungsvorgang und der sich daraus ergebenden Konsequenzen für die Therapie. H. R. Marti, Basel

F. Wuhrmann und H. H. Märki. **Dysproteinämien und Paraproteinämien.** Grundlagen, Klinik und Therapie. Benno Schwabe, Basel/Stuttgart 1963. 651 S., 201 Abb., 119 Tab., Leinen sFr 96.

Die erste, im Jahre 1947 erschienene Auflage der Bluteiweißkörper des Menschen von Wuhrmann und Wunderly war eine Pionierleistung, welche der klinischen Eiweißforschung im deutschen Sprachgebiet mächtigen Auftrieb verlieh. 1952 folgte die zweite, 1957 die dritte Auflage. Weiterhin erschien das berühmte Werk in spanischer, italienischer, englischer und französischer Übersetzung. Die durch dieses Buch eingeleitete Arbeitsrichtung, nämlich die Erforschung der Bluteiweiße des kranken Menschen mit Hilfe physikochemischer Methoden, namentlich der Elektrophorese, nahm einen enor

men Umfang an, wobei allerdings die wissenschaftliche Ausbeute während der letzten Jahre geringer wurde, indem die direkte Korrelation der Befunde mit den Krankheitsbildern das Verständnis der Pathogenesemechanismen nicht mehr wesentlich zu fördern vermochte. Erst als das komplexe Gemisch der Bluteiweiße mit Hilfe antigenanalytischer Methoden, namentlich der Immuno-Elektrophorese in homogene Einzel-Komponenten aufgetrennt werden konnte und man durch die Erforschung seltener aber typischer Krankheitsbilder wie z. B. der Agammaglobulinämie, Einsicht in den Umsatz und die Funktion einzelner Eiweiße gewann, wurden wieder nennenswerte Fortschritte im pathogenetischen Verständnis von Eiweiß-Stoffwechselstörungen erzielt. Mit der vorliegenden vierten Auflage versucht Wuhrmann zusammen mit Märki jun. dieser Entwicklung Rechnung zu tragen. Die «Dysproteinämien und Paraproteinämien» sind nicht einfach eine Neuauflage der «Bluteiweißkörper». Es handelt sich vielmehr um ein völlig neues Buch, das der heutigen Übergangssituation der klinischen Eiweißforschung voll Rechnung trägt, indem einerseits die Erkenntnisse der elektrophoretischen Ära eine umfassende Darstellung erfahren und andererseits die neuen Methoden und pathophysiologischen Erkenntnisse gebührend zur Geltung gelangen. Mit den «Dysproteinämien und Paraproteinämien» haben Wuhrmann und Märki ein Werk geschaffen, das in der Klinik wie im Laboratorium gleichermaßen unentbehrlich ist.

A. Hässig, Bern

J. C. Ressler: Groupes Sanguins et Immunisation Groupale au Congo. Editions Arscia, Bruxelles/Librairie Maloine, Paris 1963. 259 p., 66 tab.

Die vorliegende Monographie gibt einen umfassenden Überblick über die Verteilung der ABO-Blutgruppen und des Rhesusfaktors D bzw. D^u bei den verschiedenen Völkerstämmen des belgischen Kongos. Es handelt sich um einen wesentlichen Beitrag zur Blutgruppenanthropologie der schwarzen Rasse.

A. Hässig, Bern

H. Balner: Bone Marrow Transplantation after Whole-body Irradiation. An Experimental Study in the Rat. Diss. Amsterdam 1963. Drukkerij Levisson, The Hague. 134 p., 49 fig.

Es wurden homologe Transplantationen zwischen zwei Rattenstämmen nach Ganzkörperbestrahlung (750–1000 R konventionelle Röntgenstrahlen) vorgenommen und das Verhalten der Mortalität, der Hämatopoese und des immunologischen Status untersucht. Die Frühmortalität an Infekten war mit 20–40% hoch. Eine Sekundärkrankheit trat nur relativ selten auf (15–30%). Die histologischen Veränderungen, unter denen intestinale Schäden nur sehr gering waren, werden ausführlich demonstriert. Zusatz von homologen lymphatischen Zellen zum Knochenmark verstärkt die dann meist letal verlaufende Sekundärkrankheit. Der durch immunologische Techniken nachgewiesene Zustand einer hämatologischen Chimäre war bei einem Teil der überlebenden Tiere nur temporär. Wenn im weiteren Verlauf die Spenderhämatopoese wieder verschwand, so blieb trotzdem in 50% eine Immuntoleranz gegenüber der Haut des Spenderstammes erhalten. Die länger als drei Monate überlebenden Chimären zeigten eine deutlich verkürzte Lebensdauer, wobei vom 7. Monat nach der Bestrahlung ab in einem Stamm gehäuft maligne Tumoren auftraten, während in dem anderen Stamm sklerosierende Nierenveränderungen vorherrschten.

Es handelt sich um eine sorgfältige experimentelle Arbeit, durch welche die bisher meist an Mäusen gewonnenen entsprechenden Ergebnisse auch auf die Ratte erweitert wurden. Dabei ergaben sich bemerkenswerte species- und stammabhängige Unterschiede. Für experimentelle Hämatologen, Radiologen und Immunologen ist das Buch von besonderem Wert, aber wegen seiner Diskussion klinischer Fragestellungen auch für praktisch tätige Hämatologen von Interesse.

S. Witte, Erlangen

Proc. 9th Congr. europ. Soc. Haemat., Lisbon 1963; Acta haemat. 31: 129–136 (1964)

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Mechanisms for the Induction of Anisocytosis

By SATIMARU SENO

Anisocytosis is a very common phenomenon in anemia. It seems to be closely correlated with the stressed erythropoiesis in emergency cases and would be a result of an important adaptation mechanism. Until recently however the mechanism of inducing anisocytosis has escaped attention of general hematologists. In the present communication it will be demonstrated that the anisocytosis seen in anemic rabbits can be induced by the denucleation of erythroblasts at a polychromatic stage.

By drawing daily quantity of blood from adult rabbits, 20–30 ml day for 3 to 4 days, or by phenylhydrazine injection, severe anemia with marked anisocytosis may be induced one week after the initiation of the experiment. With the advance of anemia the red cell volume (MCV) increases gradually for 4 days of phenylhydrazine injection but thereafter it increases very rapidly as high as twice the original level within few days after the last injection of phenylhydrazine (fig. 1). I this stage reticulocytes often occupy more than 90% of erythrocytes. Daily observation of the cell size with advance of anemia and replace reveals an increase in the number of reticulocytes. I does not mean the gradual increase of the size of all cells but the appearance of new group of larger cells giving two peaks in PRICE JONES curves (fig. 2). The data confirm those given by BRECHER AND STOHLMAN in phenylhydrazine anemia of rats.

In the early stage of anemia the representatives of larger cell group are reticulocytes whose volume is as much as twice that of normal mature red cells, but later mature larger cells also appear and the difference between the volume of the reticulocytes and mature cells become small. But it is uncertain whether the reticulocytes mature to the same size red cells or some shrinkage occurs by *maturation*. *The big reticulocytes are fairly strong in basophilia* comparing to that of the reticulocyte from normal animal. As the size is bigger in the former its RNA contents will be enormously large, if the basophilic substance is represented by RNA.

Microspectrophotometric estimation on RNA of red blood cells of the rabbit of phenylhydrazine anemia and blood depleted

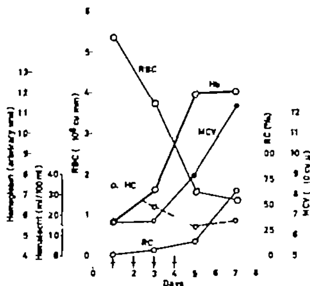


Fig. 1. Increase in hemoglobin per cell in phenylhydrazine anemia. Mean values of two rabbits. RBC: Red cell count. MCV: Mean corpuscular volume. HC: Hematocrit, RC: Reticulocyte number. Arrows show the injection of phenylhydrazine.

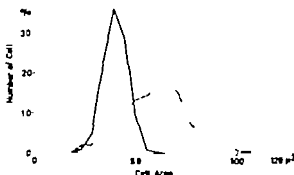


Fig. 2. Price Jones curves of peripheral red blood cells of an anemic rabbit. The animals received 4 injections of phenylhydrazine 1.5 ml of 2.5 % solution day for 4 days. The curves were drawn by observing living cells under phase contrast microscope on the blood taken before the injection and 4 days after the last injection, respectively.

	Before injection	4 days after the last injection
RBC	5.99×10^6 per cu mm	1.47×10^6 per cu mm
RC	2.1%	62.5 %
Mean diameter	7.11 μ	8.54 μ

Table I

RNA contents of reticulocytes

		Normal	Blood-depleted An.	Phenylhydrazine An.
Number of cells estimated		38	59	43
	maximum	6.3	15.5	25.0
RLaC	minimum	2.6	4.5	5.7
	mean	4.1±0.9	7.8±2.3	10.8±3.8

Values in arbitrary unit were estimated on the smeared cells stained with Azure B by Flax and Himes' method by applying two wave length method, at 590 and 520 mμ.

Normal RBC 5.74 million per cu mm, RC 2.6%
Blood-depleted An. RBC 3.77 million per cu mm, RC 58%
Phenylhydrazine An. RBC 3.24 million per cu mm, RC 85%

anemia proved a marked increase in RNA in basophilic erythrocytes, probably reticulocytes. Increasing RNA content was more marked in phenylhydrazine anemia than in blood depleted anemia (table I) This might be the difference due to the severity of anemia and seems to be related to the stage of the denucleation of erythroblasts. As it is obvious that RNA contents of erythroblasts is higher in younger cells, the high contents of RNA in reticulocytes will possibly indicate the denucleation stage of erythroblasts provided with no increase of RNA after denucleation.

To decide whether RNA increases in reticulocytes even temporarily during their maturation course or not, the change in the RNA contents have been observed in vitro on the blood from an anemic rabbit. Observations revealed that RNA decreases incessantly from the beginning in reticulocyte number showing not any transient increase. The increase in acid soluble fraction showed a marked decomposition of RNA with the maturation of reticulocytes.

The data suggest no increase of RNA in reticulocyte stage but there is a possibility that a slight synthesis of RNA is overwhelmed by a marked decomposition of RNA, which will result in incessant decrease in RNA, or in some groups of reticulocytes RNA may possibly increase. Autoradiographic method will be useful to check the RNA synthesis in each cell respectively Tritium labeled uridine was used to check the RNA synthesis of reticulocytes by in vitro incubation but the result proved that no incorporation of uridine occurs into the RNA of reticulocytes (fig 3)

Now I will show you the RNA contents of erythroblasts and reticulocytes of normal, blood depleted and phenylhydrazine in-

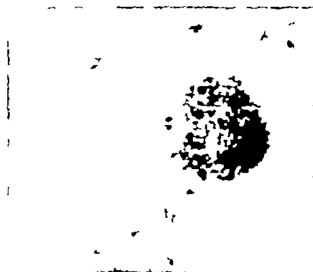


Fig. 3. The test for the incorporation of H^3-uridine into reticulocytes. No incorporation into the RNA of reticulocytes, but marked incorporation into the RNA of Ehrlich ascites tumor cell. One hour incubation.

jected rabbit (fig. 4). The level of RNA decreases with the maturation of erythroblasts showing an exponential curve. In normal rabbits the level of RNA of reticulocytes is comparable to that of orthochromatic erythroblasts but in anemic rabbits the RNA level of some reticulocytes appeared higher than that of orthochromatic erythroblasts. This tendency appeared markedly in the phenylhydrazine anemia showing the values comparable to those of polychromatic erythroblasts.

The data show that the denucleation will occur in the younger stage of maturation of erythroblasts, at least younger than polychromatic erythroblasts, though it is still uncertain exactly in what stage of the maturation of erythroblasts the denucleation is occurring because RNA in reticulocytes is incessantly decreasing after denucleation. The very stage where the denucleation is occurring can be checked by the morphologic observation of the bone marrow cells in anemic rabbits, as it is supposed that the cells in the maturation stage just before the denucleation should be highest in population on the assumption of the repeated cell division four times, from the proerythroblasts to orthochromatic erythroblasts. The observation proved that in anemic rabbits the highest population is in the basophilic erythroblasts (fig. 5) while in normal animal the

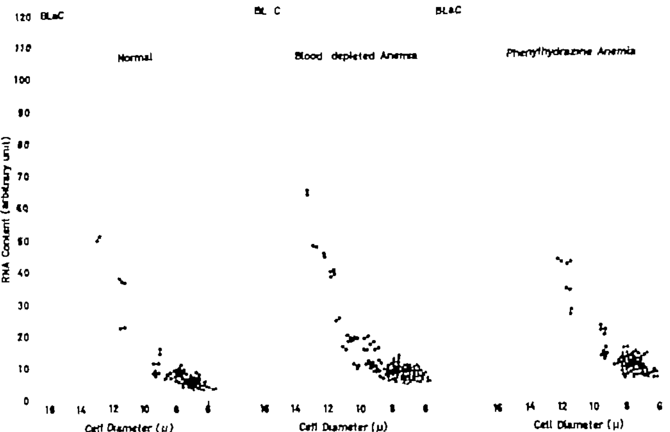


Fig 4 RNA content of erythroblasts and reticulocytes in normal and two anemic animals.

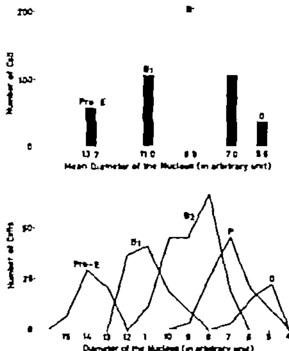


Fig. 5. Distribution of erythroblasts in each maturation stage, erythroblasts of bone marrow smear from an anemic rabbit, phenylhydrazine injection. Pro-E Proerythroblasts B_1 Basophilic erythroblasts B_2 Basophilic erythroblasts of more matured type P Polychromatic erythroblasts O Orthochromatic erythroblasts.

highest population is in the poly and orthochromatic cells. The results show that the denucleation occurs in the polychromatic stage.

Thus it is now certain that in emergency erythropoiesis the denucleation can occur at the polychromatic stage of erythroblasts and the big and RNA rich reticulocytes are formed. But an important problem is that such reticulocytes born of the abnormal denucleation in the early stage of maturation can synthesize hemoglobin in a normal way. As it is supposed that the synthesis of hemoglobin, the species specific protein, is genetically controlled by nuclear DNA probably through the mediators like messenger RNA, the mediator should be fully developed and transferred into the cytoplasm before the denucleation in order to synthesize hemoglobin in normal way as in normally denucleated cells.

Microspectrophotometric determination of hemoglobin contents per cell in the blood depleted rabbits showed somewhat low

levels in both mature cells and reticulocytes comparing to those in normal animals, respectively This seems to show the reticulocytes produced by denucleation in polychromatic stage are inferior in the ability of hemoglobin synthesis to those produced by normal denucleation. This might be due to denucleation at the stage of incompleted mediator transfer into the cytoplasm of erythroblasts. But in blood depleted anemia the materials for hemoglobin synthesis especially iron, is deficient and this iron deficiency may be responsible for the production of hypochromic erythrocytes.

Observation of phenylhydrazine anemia, where most of iron freed from red cells by hemolysis is stored and re-utilized proved very high levels of hemoglobin in erythrocytes (fig 1) This shows that the reticulocytes formed by early denucleation are already well equipped with mediator for hemoglobin synthesis as normal reticulocytes, i. e. the cytoplasm of erythroblasts has been fully differentiated to produce hemoglobin already by the stage of polychromatic erythroblasts. The fact well explains that some of the large erythrocytes and reticulocytes have a quantity of hemoglobin with the apparatus for hemoglobin synthesis in double in their large capacity formed by skipping the last cell division to the orthochromatic stage. Consequently the last cell division from polychromatic to orthochromatic erythroblasts in normal animals will effect only to enlarge the surface area by which the efficiency of oxygenation per cell unit of hemoglobin will be increased but not the synthetic ability of hemoglobin.

Now I will summarize the results obtained. In emergency case the denucleation of erythroblasts can occur at the stage of polychromatic erythroblasts. The denucleated cells at the polychromatic stage have a large volume twice the normal, consequently anisocytosis occurs. The big reticulocytes are frequently higher in RNA level than that of orthochromatic erythroblasts and can synthesize hemoglobin twice the normal provided with materials under the appropriate conditions.

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The Erythropoietic Porphyrias

By L. Heilmeyer

It had been the great merit of Watson and R. Schmid to have shown that porphyrias are no universal metabolic disorders, such as e. g. diabetes mellitus, but that the disturbance of the metabolism is restricted to certain organs, i. e. the liver and the bone marrow. Therefore we distinguish between hepatic and erythropoietic prophyrias. The latter are the expression of certain enzyme disorders in hemesynthesis within the erythrocytes. Thus erythropoietic porphyrias have become part of the field of research of hematology and it seems justified to give a report on this within the frame of this congress. Up to the present day we distinguish three different forms of erythropoietic porphyria.

1. *Porphyria congenita chronica (Morbus Günther)*

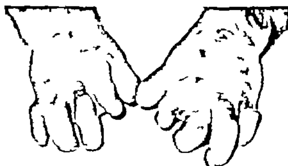
We have had two cases of Morbus Günther recently under our observation. The first case, a young man, aged 22, had already from childhood on a red urine. His sensitivity to light was observed when he was three years of age. Finally formation of blisters and scars occurred on the spots of skin which were exposed to light, as well as his teeth got a red coloration. Figure 1–3 demonstrate the severe alterations in face and hands. Erythrokinetics show an abbreviation of erythrocyte life-span to ⅓ of the normal value at the average with significant deviation of the lifetime of the individual cells. Above all a very short-lived share among them is detected. In the bone marrow numerous porphyroblasts and porphyrocytes were found. Electronoptically cells being in decay were particularly striking, they probably were erythroblasts which are supposed to be the expression of an ineffective erythropoiesis (fig. 4). Subsequent to splenectomy a normalisation of the erythrocyte life-span occurred as well as porphyrin excretion was improved.





1 3

Figs 1 3. Alterations on hands and face in one case of erythropoietic porphyria (see text)



2



Fig. 4 Electronoptical picture of erythroblasts in the bone marrow D = Erythroblast in decay S = Ferritin from destroyed Hb. EB = Erythroblasts (by courtesy of Dr Mölbert, Freiburg/Br.)

The second case was a boy aged five. He showed delicate scar formation only in the face. On his hands he had blisters and necroses (fig. 5 and 6). The conditions of porphyrin metabolism are of particular interest. Figure 7 shows the pattern of hemeprecursors of both the cases mentioned. A strong increase of uroporphyrin is emphatic in the erythrocytes, it is followed by a less significant increase of coproporphyrin and a still smaller increase of protoporphyrin. Hemesynthesis, however, was quantitatively sufficient in the final effect as resulted from the normal content of hemoglobin and erythrocytes in the blood. Thus anemia was absent. A somewhat increased hemolysis was present (28‰ of reticulocytes) which was compensated, however. There were also no pathological sideroblasts in the bone marrow. Normal sideroblasts were not increased thus indicating a sufficient hemesynthesis. In contrast a severe qualitative disorder of porphyrin synthesis was present in both cases.

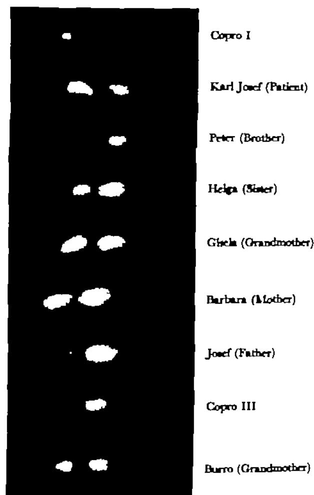


Fig 10. Analysis of coproporphyrin isomers in erythrocytes of the different members of family 2. (Paper chromatography in 2,6-Lutidin water.)

which is however present in a much smaller quantitative extent, so that the relatives of this family seem to be completely healthy Evidently these findings concern heterozygote bearers of hereditary marks, who are phenotypically healthy persons. Figures 8 and 9 will demonstrate these relations quite clearly The numbers represent the amount of uroporphyrin which were established in the erythrocytes respectively The normal upper limit in our method is 4 micrograms/100 ml of erythrocytes. The isomer analysis of the coproporphyrin found in the erythrocytes of the singular family members revealed a remarkable portion of type I

isomer in all cases with one exception, i. e. the genotypically healthy brother Peter (fig 10) As seen on those figures there is also a recessive hereditary succession present. Thus it was possible for the first time to clarify the hereditary succession of the disease by grasping the heterozygote bearer of hereditary marks. The clinical picture of this disease only appears in homozygoty In the case of Po. we were able to detect in addition that father and mother are relatives. They both possess a common greatgrandfather they thus are cousins in the second degree.

2 Protoporphyrinaemic actinodermatosis

In addition to Günther's porphyria another erythropoietic form of porphyria has been known since 1953 the genesis of which is, however completely different. For the first time it was described by KOSENOW AND TREIBS in 1953 Further cases were communicated by KOSENOW in 1954 and later by LANGHOF et al. in 1961 MAGNUS et al. described the same picture of disease in 1961 under the name of erythropoietic protoporphyria. Here too, a congenital disease is concerned, having been observed in brothers and sisters for several times. It commences already in early childhood. Subsequent to exposure to sunlight a swelling occurs at the exposed spots or erythema and formation of urticaria is observed. In that family published by LANGHOF 4 of 9 sisters and brothers experienced regularly after solar radiation strongly itching skin wheals. In contrast to Günther's disease, however red urine is never excreted and the teeth are never coloured red. Anemia is usually also absent. Serum iron is mostly normal. In two brothers, as described by LANGHOF erythrocyte and hemoglobin values were even above normal values. In blood as well as in the bone marrow bright fluorescent erythrocytes and erythroblasts are evident. In contrast to porphyrocytes in Günther's disease, however red fluorescence becomes extinct after short exposure to light as is typical for the fluorescence of protoporphyrin. TREIBS was already able to establish by chemical methods that these erythrocytes contain protoporphyrin in an increased amount. In addition to the acute phenomena of photourticaria, to the release of which solar radiation of some minutes is already sufficient LANGHOF et al. also found signs of chronical damage by light in form of colloidmilia and hyperkeratosis. A piece of extirpated skin shows an intensive red brown fluorescence in microscopic cut.

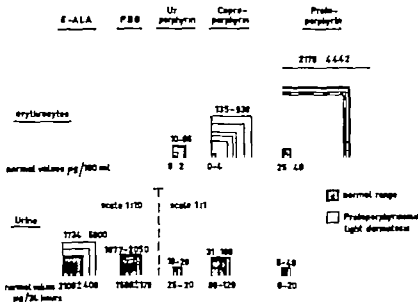


Fig 11 Hemprecursors in erythrocytes and in urine in protoporphyrinemic light dermatosis (4 cases)

Together with Clotten the blood of two brothers, whose clinical findings were published by Langhof could be studied concerning its porphyrin metabolism. Fig 11 shows the pattern of hemeprecursors of all cases examined by us. Particularly the excessively high value for free erythrocyte protoporphyrin is striking which corresponds to the 50 to 100-fold of the normal. Such elevated erythrocyte protoporphyrin values have never been found in any other diseases up to the present. The denomination erythropoietic protoporphyria as well as protoporphyrinemic actinodermatosis are thus both justified. Simultaneously also erythrocyte coproporphyrin and erythrocyte uroporphyrin are increased as is frequently seen in significantly elevated protoporphyrin values. It seems quite understandable that with such increased porphyrin values in the erythrocytes also larger amounts migrate into plasma and thus into the organism, thereby effecting photosensibilisation According to studies of Philippi the photosensitizing effect of protoporphyrin is known. It is, however far weaker than that of uroporphyrin I. Therefore no severe burnings and mutilations occur in these patients. The findings in the urine show only slight deviation in contrast to the blood findings. Excretion of uroporphyrin and coproporphyrin is only very slightly increased. In contrast Brugsch de-

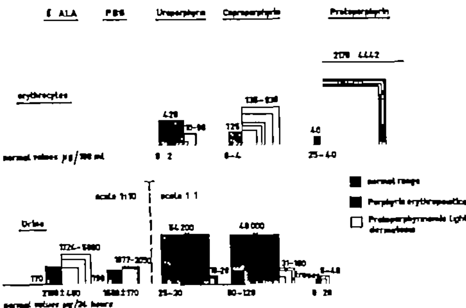


Fig. 12. Porphyrins of erythrocytes and urine in erythropoietic porphyria and in protoporphyrinaemic light dermatosis.
Case of Dr. STICH.

tected in the faeces of such cases a very high protoporphyrin excretion which was about the 50-fold of normal values. In the incubation test with blood of such a case which was handed to us by Prof. LANGHOF from Jena an extremely decreased porphyrin synthesis was present which only amounted to 3 / of the added δ-ALA. This enormous inhibition of the synthesis might be the consequence of the vast amount of protoporphyrin in the erythrocytes, as we could prove that addition of protoporphyrin to the hemolysate regularly inhibits porphyrin synthesis. The interpretation of these findings is rather difficult and at present still impossible. The vast increase of protoporphyrin cannot be due to a block in hemsynthesis, as we could not find any increase of serum iron and any increase of sideroblasts in the bone-marrow. Anemia is also absent. Perhaps there could be present a disorder in the relation of protoporphyrin to protoporphyrinogen. But perhaps there could also be present a disorder in the degradation of protoporphyrin. These questions are still completely obscure and need further investigation. However it is certain that the kind of disorder of porphyrin metabolism is completely different from that present in Günther's porphyria.

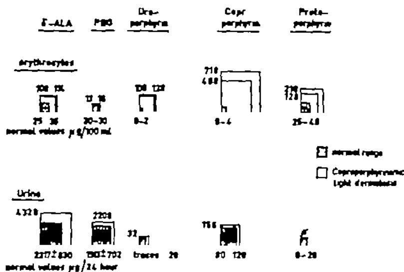


Fig 13 Coproporphyrinemic light dermatosis. Hemeprecursors in erythrocytes and in urine.

The porphyrins found in protoporphyrinemic actinodermatosis all belong to the normal isomeric type III Fig 12 demonstrates the findings of both diseases in comparison The difference of both is thereby clearly seen.

3 Coproporphyrinemia

A third up to the present unknown form of congenital erythropoietic porphyria, also with slight photosensibility could be detected by us recently A bank employee, aged 22 is the subject of our observations who suffers from evident hypersensitivity of the skin after exposure to light since her 10th year of age. Subsequent to stronger solar radiation, particularly in winter when skiing, severe swellings occur prevailingly in the face and at the arms, lesser at the legs. Strong itching and burning accompany these swellings. Usually they appear many hours after exposure to light, frequently only in the evening and are healing up under formation of scales. Other diseases were not suffered from by her *One daughter of her father's brother is said to suffer from similar photosensitivity*

The blood picture of this patient was practically always normal. There was only a slight reticulocytosis of 23‰ present. Serum iron was 84 micrograms. Bilirubin 0 9 mg %. There was no increase of sideroblasts in the bone marrow The pattern of hemeprecursors of this case is shown by the following picture 13

As is seen, there is an enormous increase of erythrocyte coproporphyrin which reaches up to 200-fold of the normal. Erythrocyte uroporphyrin is also significantly increased, whereas free erythrocyte protoporphyrin amounts only to about 6-fold of the normal values. There was also a slight increase of δ-aminolevulic acid in the urine. In contrast we detected a slight increase of protoporphyrin in the faeces. An incubation test with the hemolysate of the blood of this patient demonstrated a significant inhibition of porphyrinsynthesis, the cause of this being probably the large amount of preformed coproporphyrin in the erythrocytes. However the formation of protoporphyrin was increased. All these findings, as well as the clinical picture, show that there is a disease present here which is different from the protoporphyrinemic actinodermatosis. There is no formation of urticaria. Coproporphyrin is largely increased in the blood, protoporphyrin is only very slightly increased. Signs of a chronic photosensibilisation are also absent. It seems that in this form of disease photosensibilisation is less marked when compared to all other congenital erythropoietic porphyrias. It does not induce symptoms of disease by normal solar radiation but only after strong exposure to sunlight.

I hope to have shown that essential progress was made in the field of erythropoietic porphyrias during the last years. Nature of the disorders could be better clarified in Günther's porphyria, even if we are still far away from a final elucidation. We succeeded for the first time in clarifying the hereditary mode of this disease by grasping the heterozygote bearers of the marks. Further Kosenow's protoporphyrinemic actinodermatosis could be demonstrated as a peculiar form of erythropoietic porphyria. And we further succeeded in detecting a third form of erythropoietic porphyria with the coproporphyrinemic photosensibility. I am hoping that these interesting disorders, the cause of which is due to deviation of enzymes serving the hemsynthesis in the erythrocytes, will become still more interesting to the hematologist than up to now. For all the pictures of disease mentioned here are certainly belonging to the field of hematologic research.

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Clinical Assessment of Granulopoiesis

By A. Fieschi and C. Sacchetti

This report summarizes the recent findings with respect to the kinetics and the pathophysiology of neutrophilic granulocytes. It has been known since many years that the circulation of granulocytes is not comparable with the circulation of erythrocytes. The marginal flow the adherence to capillary beds and the visceral sequestration were well demonstrated (1 2 8–10 41) However the clinical study of the granulopoiesis did not progress until refined methods became available for labeling and quantitating granulocytes in a physiological way without altering the state of the recipient or damaging the labeled cells (3–7 26 29 30 31–40)

Granulocyte kinetics in blood

The autotransfusion of granulocytes labeled in plastic bags with DFP[32] showed that there are two subunits forming the total blood granulocyte pool (T G P) the circulating granulocyte pool (C.G.P) and the marginal granulocyte pool (M G P) The marginal pool is represented by cells adhering to the capillary and venular beds, and *it approximately equals the number freely circulating*

The circulating and the marginal cells are in continuous interchange. The ratio between the two pools in the whole body is stable in the steady state, but the degree of the mobilization from the marginal pool varies with a host of factors rate of blood flow pressure, tachycardia, chilliness, muscular exercise, adrenalin, all causing leukocytosis. In reverse, factors like collapse or histamine facilitate the margination and the appearance of leukopenia. These conditions of leukocytosis and leukopenia are of short duration and do not modify the effective number of granulocytes in blood. *They are* apparent leukocytosis or leukopenia indeed, and we understand

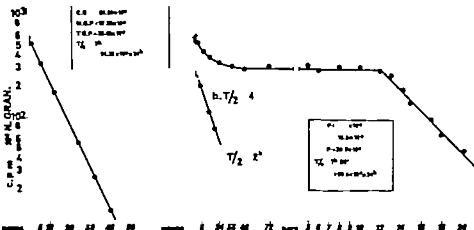


Fig 1. Normal subject. Autotransfusion of N. Granulocytes labelled with DFP^{32} in plastic bag (curve on the links) Same case N Granulocytes labelled administering DFP^{32} intravenously (curve in the right).

how the traditional counting of the leukocytes can easily offer only a partial sight of the real number of the granulocytes in the blood.

The neutrophilic granulocytes do not spend all their life in blood but leave the circulation and reach the tissues, where granulocytes perform most of their defensive role. There is abundant evidence that granulocytes in the blood are expendable cells that in a random fashion cross the capillary endothelium and, once into the tissues, do not re-enter the circulation, almost in a detectable number (18 19 23 26)

These facts simplify the interpretation of kinetic data, because we can study the granulocytes turnover in the blood and in the bone marrow separately from the granulocyte life span in the tissues. However we must point out that the degree of granulocyte demand into the tissues modifies the turnover rate of the cells in the blood and accelerates the delivery of new cells from the bone marrow

In the physiological steady state the half time disappearance of mature granulocytes from the blood is of about 7 hours. The complete renewal of the total blood granulocyte pool takes about 48 hours. Fig 1 reports an example of the granulocyte kinetic carried out by means of autotransfusion of granulocytes labeled with DFP^{32}

Granulocytic kinetics in the bone marrow

Granulopoiesis in the bone marrow can be considered as constituted of 4 compartments, each one with particular functional properties

1 The stem cell compartment, which differentiates into granuloblasts (myeloblasts) The stem cells assure the renewal of the granuloblastic population, responding to stimuli which can induce hyperplasia.

2 The proliferating cell compartment, including myeloblasts, promyelocytes and about the 75 / of myelocytes. These cells proliferate and give rise to the granuloblastic bulk.

3 The maturative compartment, including the cells that never proliferate but only mature a fraction of the myelocytes and the metamyelocytes.

4 The reserve compartment or marrow granulocytes reserve (M.G.R.) a mass of mature granulocytes, which provides a readily available supply of cells to be released into the peripheral blood upon demand. The M.G.R. is a particular entity of the granulopoiesis large about 20 times the mass of the granulocytes in the blood (4 5 7 17-21 23 24 26, 35 40) In normal subjects the M.G R. is replaced about every 6 days during this time, it releases mature cells at a rate sufficient to mantain the normal granulocyte counting in the blood even if the granuloblast proliferation in the marrow is stopped.

The bone marrow compartments can be recognized by means of several labelling techniques, using tritiated thymidine, P^{32} or S^{35} compounds, but as to our experience the label with DFP^{32} 'in vivo gives the best results in the study of the human casuistics (4, 14, 17 22 30, 33 42)

In the fig 1 is reported an example of the DFP^{32} curve 'in vivo in physiological steady state. The first phase of the curve is related to the turnover of the granulocytes in the blood the second phase concerns the granulocytes delivered from the marrow reserve and the maturative compartment, the third phase concerns the cells originated in the proliferative compartment of the marrow

Bone marrow and blood granulocyte interrelationships

Leukocytosis may result from mobilization of the marginated cells as after exercise or adrenalin administration (6, 7 26, 30).

Moreover leukocytosis induced by leukopheresis and other depletion techniques is supported by the marrow granulocyte reserve and does not depend upon cell proliferation (12, 15–21) The M.G.R. indeed satisfies the immediate demand of granulocytes In the meantime the increased removal or utilization of granulocytes is in turn compensated by means of augmented production by the staminal cells, which allows the compensatory granuloblastic hyperplasia. However the compensatory mechanism does not become manifest for a period of several days (5–8 days in humans) (16, 17 26 34 40) During this period of active marrow response, a sustained peripheral demand could overcome the replacement capacity of the precursor cell pool and leukopenia would appear and persist. However according to the degree of the compensation and to the disponibility of the M.G.R. it is possible to maintain a balance, which is not necessarily reflected by the peripheral blood concentration of granulocytes. In such a way we may observe normal counting values of granulocytes in the blood, while the marrow approximates a failure that would be suddenly manifest if a new increase of the demand supervenes. Between the granuloblastic hyperplasia, with a bulk of M G.R. of normal size (corresponding to a condition of complete compensation) and the hypoplasia with depleted M.G.R., we can recognize a number of conditions in which the compensatory mechanism is very similar to that occurring in the erythropoietic response the only difference is that in the granuloblastic hyperplasia a M.G.R. of various degree is present.

We must point out that leukopenia is not necessarily due to profound hypoplasia. An hyperplasia of various degree is often present This latter condition, however can be a transition phase to the true hypoplasia.

We can differentiate a first type of hypoplasia in which the demand or the loss of the peripheral granulocytes predominates and a second type, in which the utilization of granulocytes is within normal limits and the leukopenia may be considered myelogenous in origin. These conditions belong to a staminal defect, constitutional or acquired, or to a primary damage of the proliferating cells due to chemical or physical factors. It appears evident that the differentiation between the various types of leukopenia requires the evaluation of the peripheral granulocyte turnover and of the kinetic of the bone marrow populations.

By means of chemotherapeutic agents or irradiation, the development of the hypoplasia and the successive recovery can be studied. The damage develops in the following phases 1) decrease or arrest of the proliferation 2) progressive depletion of M.G.R. 3) progressive depletion of the bone marrow granuloblasts 4) granulocytopenia in the blood. When recovery is possible, the rise in blood granulocytes precedes the rebuilding of the M.G.R. In more severe conditions, the recovery of the M.G R. appears independently from the full efficiency of the precursor pool. The stem cell impairment can reduce, for a long while, the degree of the compensatory response in course of an increased demand of granulocytes (25, 26 31 34 40)

The early recovery phase after irradiation shows an example of leukopenia with hyperplastic immature marrow similar to that described as maturations arrest In this instance, as in more frequent condition found in agranulocytosis, we never can demonstrate a cellular defect by means of "in vivo and in vitro studies the bone marrow cells appear to proliferate and to maturate in a normal way Therefore, we consider the myeloblastic promyelocytic marrow to correspond for the most cases with the delivery of the previously inhibited stem cells.

A serious prognostic value is related to the myelocytic marrow in which the M.G.R. is depleted and the staminal pool is inhibited.

Patterns of arrest of the mobilization of the mature cells from the bone marrow are also described. This morphological interpretation does not seem to be definitely proved up to now Considering that the splenomegalic patterns of altered delivery of mature cells are available, we can recognize two different situations (39 40) The former shows a prevailing granulocytic sequestration with a marginal granulocyte pool larger than normal, mobilized by adrenalin (fig 2) In these cases leukopenia is only apparent and the M.G.R. is normal. A mild anaemia is often present and it is important to note that also anaemia, like leukopenia, is only apparent. The fact that the total granulocyte pool does not exceed the normal limits (although the circulating granulocyte pool is reduced in size) and the M.G.R. is of normal size, indicates that the regulation of the delivery of the mature cells from the marrow is not influenced by the number of the circulating granulocytes, but by the real cellular bulk in blood whatsoever distributed.

Fig 2. Splenomegaly with prevailing granulocytic sequestration. Autotransfusion of N. Granulocytes labelled with DFP^{32} in plastic bag (curve on the left). Same case N. Granulocytes labelled with DFP^{32} intravenously (curve on the right).

The latter condition is characterized by the prevailing utilization (or destruction) of granulocytes in respect to the sequestration. In these cases, the total granulocyte pool in the blood is normal or reduced under the normal values, but the granulocyte turnover rate is very augmented. The M.G.R. may be impaired and it preludes insufficiency (fig 3)

Criteria for the evaluation of the granulopoiesis

The evaluation of granulopoiesis is a study of the granulocyte kinetics. The most important data refer to the degree of the granulocyte utilization, performed by the blood granulocyte turnover rate (G T.R.) referred to the total blood granulocyte pool, and to the evaluation of the marrow granulocyte reserve (M.G.R.) Both these values considered together offer the possibility of more precise information independent from the peripheral blood concentration of granulocytes.

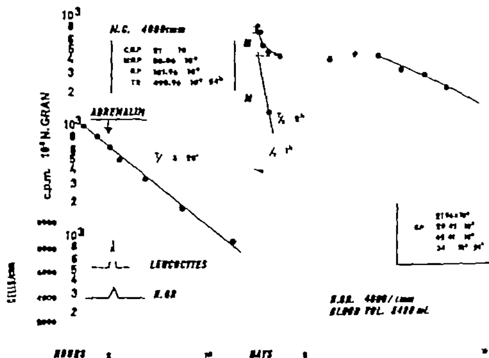


Fig 2. Splenomegaly with prevailing destruction of granulocytes. Autotransfusion of N Granulocytes labelled with DFP in plastic bag (curve on the left) Same case N Granulocytes labelled with DFP intravenously (curve on the right)

For clinical purposes, we use a group of simple screening tests that do not require extensive laboratory studies and can be realized in a very short time 1) total and differential counting of the leukocytes 2) Arneth s classification of granulocytes 3) mobilization of the marrow granulocyte reserve (M.G R.) (test with Pyrexal) 4) morphologic examination of the bone marrow (bone marrow puncture)

If the above tests do not offer satisfactory interpretative data, the granulocytic kinetics must be studied by means of labeled granulocytes. According to ATHENS et al. we found in the DFP the best label for clinical investigations.

The *tests reliable with DFP* concern (5 7 26, 30, 40)

1) Autotransfusion of labeled granulocytes in plastic bag, by which it is possible to measure the total blood granulocyte pool (T G.P) the circulating granulocyte pool (C.G P) the marginal granulocyte pool (M.G.P) the half time disappearance (T ½) the granulocyte turnover rate (G T.R.)

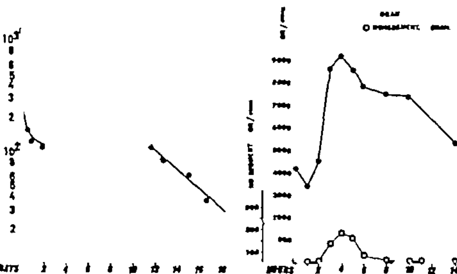


Fig 4 Normal subject. Granulocytes labelled *in vitro* with DFP[32] intravenously: the curve shows platean of normal length (curve on the left). Mobilization of the M.G. Reserve using the Pyrexal test: high increase in the circulating granulocytes (curve on the right)

The G T.R. per 24 hours, referred to the T ½ and to T G.P., offers an evaluation of the granulocyte removal Figures 2 and 3 give an example of a normal and of an augmented utilization of granulocytes. In splenomegalic conditions, the autotransfusion may give *erroneous results because of the sequestration. They can be* avoided provoking mobilization of the infused cells by means of adrenalin (fig 2) or performing the labeling in vivo

2) Labeling "in vivo of the granulocytes in the blood and in the bone *marrow administering DFP[32] intravenously In such a* way all the dinamic pools of the blood as well as the reserve of the marrow can be calculated, but it takes several days (fig. 1)

As to the interpretation of the simplest tests attainable without the use *of radioisotopes, there is abundant evidence that the intra*venous administration of bacterial endotoxin (Pyrexal a lipopolysaccharide from Salmonella aborts equi) (17 19 21 26, 28, 35 37) in doses of 0 1 gamma provokes a sustained neutrophilic granulocytosis due to the release of the granulocytes from the M.G.R. The degree of the leukocytosis agrees well enough with the dispoaibility of the M.G.R., as shown in fig 4 and 5 in which the response to Pyrexal has been controlled by the in vivo labeling

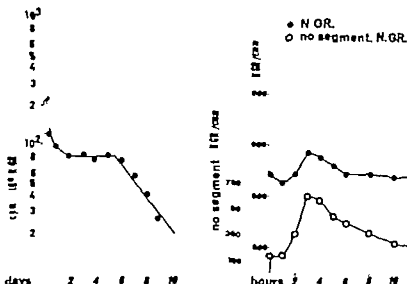


Fig. 5. Hodgkin's disease 30 days after X-irradiation (7,000) without granulocytopenia in the blood. Granulocytes labelled "in vitro" with DFP[32] intravenously: the curve shows plateau shorter than normal (curve on the left). Mobilization of the M.G. Reserve using the Pyrexal test: poor increase in the number of circulating granulocytes with an high increase of non-segmented granulocytes (curve on the right).

with DFP[32] The response is studied by timing the evaluation of the absolute number of granulocytes in the peripheral blood and performing the Arneth's classification. A characteristic of the normal response is the absence of a significative 'left shift' in the granulocytes. This fact suggests that the M.G.R. releases granulocytes in an orderly way according to their age. Therefore, some refinements in the interpretation of the Pyrexal test as well as in the Arneth's classification can be proposed.

Independently from the number of the circulating granulocytes, the 'left shift' indicates that the M.G.R. is reduced in size. The bone marrow production of granulocytes may be active but without a complete compensation, or may be reduced and inadequate also for a peripheral demand not exceeding the normal degree.

The Pyrexal test perfectionates these preliminary considerations and gives an evaluation about the size of the M.G.R. a) if the induced leukocytosis reaches, within about 4 hours, a value of 200% (min. val. 170%) the M.G.R. is of normal size and supported by a good compensation b) if the response to Pyrexal gives a defective or even absent leukocytosis and the Arneth's formula shows a 'left

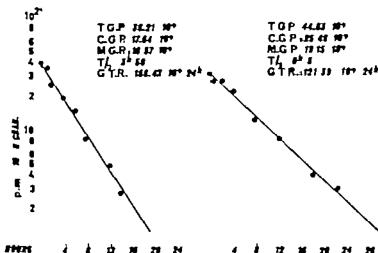


Fig. 6. Lupus e.d. Autotransfusion of N Granulocytes labelled with DFP[32] in plastic bag. Before therapy (curve on the left). 30 days after prednison 40 mg p d. (curve on the right).

shift with nonsegmented neutrophils over the 4 / (to be calculated in absolute number) the M G.R. is reduced in size and the granulopoiesis is impaired.

In leukopenic patients, the most indicative value is not due to the per cent increase in the number of circulating cells, but to the degree of the left shift.

Some applications concerning prognosis and therapy

If we consider the protective role of the M.G R. it is clear that several pathologic conditions can develop for a long time without showing definite changes in the number of circulating granulocytes. For the same reason the complete recovery is difficult to be recognized.

The Arneth s classification and the test for the mobilization of the M.G.R. can support a positive mean. In particular it is possible to precise the degree of tolerance for subjects working in un favourable conditions (benzol, X-irradiation) and to ascertain the true recovery

The evaluation of the integrity of the M.G.R. is also useful in order to control the effects of chemotherapeutic compounds and of X therapy as well as in planning the rest duration between successive cycles of therapy

The demonstration of an excessive loss of granulocytes (high G T.R.) suggests the opportunity of more detailed analysis in assessing the causes (for instance, the research of anti-granulocytic factors) Subsequently it may be important to know the effects of the therapy

Corticosteroids are known to induce neutrophilia with an increase of the total blood granulocyte pool, chiefly by a reduced granulocytic utilization (7 13 27 38) In this concern, the autotransfusion of granulocytes labeled with DFP[32] gives evidence of the dose-effect relationship during the administration of adrenal steroids (fig 6) When therapy is discontinued, the autotransfusion of labeled granulocytes makes it also possible to control the effectiveness of the improvement, while the blood cell count alone may be source of errors.

Recent progresses in the study of the granulopoiesis do not indeed diminish the value of the morphological methods which are necessary for a direct recognition of the bone marrow cytological composition.

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Evolution et traitement des purpuras thrombopéniques idiopathiques

Par Jean Bernard

Il est facile de reconnaître un purpura thrombopénique idiopathique, malaisé de prévoir son évolution, plus difficile encore de fixer les règles de son traitement.

Le présent travail, entrepris par Mme M. J. Larrieu, MM. J. Caen, G. Meshaka et nous-même, a pour objet l'examen de ces difficultés. Plus exactement nous avons tenté de répondre aux questions posées:

1. Peut-on prévoir la durée d'un purpura thrombopénique idiopathique récent?
2. Existe-t-il des purpuras thrombopéniques idiopathiques de très long cours?
3. Que sont les purpuras thrombopéniques idiopathiques intermittents?
4. Quelles sont les raisons de l'éventuelle évolution fatale? sa fréquence, son moment?
5. Que peut-on attendre de la cortisone?
6. Que peut-on attendre de la splénectomie?

Matériel et méthodes

Notre étude est fondée sur l'analyse de 204 cas de thrombopénies idiopathiques recueillies entre 1952 et 1962. De ces 204 observations, 146 ont pu être utilisées.

Les thrombopénies de cause connue ont été soigneusement écartées. Dans tous les cas la moelle était équilibrée, non leucémique, et contenait, au moins aux premiers examens, un nombre normal ou supérieur à la normale de mégacaryocytes.

Aux méthodes habituelles d'étude de l'hémostase ont été jointes dans la majorité des cas une étude isotopique des plaquettes marquées (Najean), une étude immuno-hématologique (Dausset).

L'étude des plaquettes marquées au radio-chrome (NAJEAN) a été faite dans 113 cas. Cette méthode est actuellement doublement limitée

1 Elle ne peut (en-dessous du chiffre de 80.000 plaquettes) être faite qu en isotransfusion et postule que la durée de vie des plaquettes étrangères est peu différente de celle des plaquettes du malade.

2 Elle demande que le malade ait été peu ou pas transfusé puisque l'installation d une iso-immunisation est sans parallélisme exact avec le nombre des transfusions.

En dépit de ces nécessaires réserves, elle donne des résultats dont la constance mérite d être soulignée.

1 *Alors que la* durée de vie des plaquettes chez *les* sujets normaux, dans les thrombopénies idiopathiques guéries, dans les thrombopénies par insuffisance médullaire, est de 7,5 à 11 jours avec une moyenne de 9 1 jours, cette durée de vie est constamment diminuée dans les thrombopénies idiopathiques à moelle normale en période évolutive. Il existe une corrélation entre le chiffre des plaquettes circulantes et la durée de vie des plaquettes étrangères ou personnelles.

La durée moyenne est de un jour pour un chiffre de plaquettes inférieur à 50 000 par mm³ de 1 4 jour pour un chiffre de plaquettes variant entre 50 000 et 80 000 de 2,3 jours entre 80.000 et 120.000

La cytopénie du purpura thrombopénique idiopathique paraît essentiellement liée à l'hyperdestruction des plaquettes constatée dans tous les cas sans exception.

Les thérapeutiques actives restaurent une survie normale ou subnormale des plaquettes.

2 *La production médullaire des plaquettes*, qu il n est pas facile de calculer (car la survie est courte) avec précision, ne paraît pas réellement augmentée (en dépit de l apparente hypermégacaryocytose)

3 L étude des plaquettes marquées permet, on y reviendra, de préciser les indications des thérapeutiques, d apprécier leurs effets.

4 Il n existe pas de corrélation rigoureuse entre la durée de vie des plaquettes et les résultats de l'examen immunologique.

L étude isotopique prouve ainsi l unité du mécanisme de la thrombopénie idiopathique ni la mode de début, ni l allure évolu-

Saison. La presque totalité des formes aiguës se répartit entre deux périodes de l'année, hiver et printemps. La répartition des autres formes est dispersée.

Début. Une thrombopénie chronique peut commencer brusquement, mais la soudaineté du début est plus fréquente dans les formes aiguës.

Clinique. Le syndrome de thrombopénie aiguë avec bulles sanglantes buccales et hématurie, que nous avions isolé en 1952, est très remarquable par sa ressemblance avec l'onyalai Sud-Africain. Il n'est pas spécifique. Les bulles sanglantes buccales sont cependant beaucoup plus fréquentes dans les formes aiguës que dans les formes chroniques. Ce fait mis à part, il ne paraît pas exister de différences cliniques entre le purpura thrombopénique aigu et la première période d'une forme chronique.

Hémostase. De même l'étude de l'hémostase ne révèle pas de différence.

Étude isotopique. La durée de vie des plaquettes, leurs lieux de séquestration sont les mêmes dans les deux cas. Nous n'avons pas noté d'influence de l'ancienneté de la thrombopénie sur la durée de vie des plaquettes.

Étude immuno-hématologique. L'étude immuno-hématologique ne montre pas de différence nette. Tout au plus peut-on noter que les réactions immunologiques sont un peu moins souvent positives pour les purpuras aigus des enfants.

Ainsi, il est très difficile, sinon impossible, de discerner si une thrombopénie récente va évoluer sur un mode aigu ou chronique. La fréquence plus grande chez les jeunes, la fréquence plus grande en certaines saisons, la fréquence plus grande des débuts soudains, la fréquence plus grande des bulles buccales, la fréquence moins grande des réponses immunologiques positives sont des caractères des thrombopénies aiguës. Aucun de ces caractères n'a de valeur formelle.

L'évolution du nombre des plaquettes peut permettre quelques précisions utiles.

1. Si ce nombre des plaquettes n'a pas atteint 100.000 après trois semaines, la guérison en un mois ne peut [illegible] être espérée.

2. Si le nombre des plaquettes n'a pas attein[illegible] au 60ème jour, il faut s'attendre à [illegible]ution longue [illegible] robable[illegible]ment six mois.

tive, ni la présence ou l absence d anticorps décelable, ni l efficacité inégale des thérapeutiques ne séparent des formes de mécanisme différent.

Immunologie. Nous n insisterons pas sur les réactions d agglutination rares à 37 assez fréquentes à 4 mais observées dans de nombreuses autres affections et bien difficiles à interpréter Nous soulignerons l'intérêt des méthodes de consommation de l antiglobuline sur les propres plaquettes du malade (test direct) (Moulinier, Steffen) ou sur les plaquettes étrangères préalablement sensibilisées par le sérum du malade (test indirect) (Dausset)

Ces tests sont positifs dans la moitié des cas environ de thrombopénie idiopathique. Ils sont rarement positifs en dehors de ce syndrome, mais ont pourtant été signalés dans le lupus érythémateux disséminé, dans certaines pancytopénies chroniques idiopathiques, dans certaines thrombopénies secondaires à une affection maligne du tissu lymphoréticulaire.

Il n a pas été possible jusqu à présent de noter de différence nette entre les thrombopénies idiopathiques à test positif et celles à test négatif. Tout au plus peut-on noter 1) la plus grande fréquence des formes à test négatif chez l enfant, 2) la gravité un peu plus grande des formes à test positif.

Peut-être cette insuffisante discrimination est-elle due à l insuffisance des techniques de l épreuve de consommation de l antiglobuline, à la complexité récemment reconnue des antiglobulines individuelles.

Peut-on prévoir la durée d'un purpura thrombopénique récent? Un purpura thrombopénique idiopathique vient d être reconnu. Quelle va être sa durée? Peut-on espérer une évolution courte? Doit-on redouter une évolution longue? Nous avons arbitrairement classé les thrombopénies idiopathiques en quatre groupes aiguës évolution totale inférieure ou égale à un mois subaiguës évolution allant de un à trois mois chroniques évolution supérieure à trois mois intermittentes. L étude comparée des caractères étiologiques cliniques et biologiques de ces groupes donne les résultats suivants

Age et sexe. Les formes aiguës, subaiguës et chroniques sont observées à tout âge les formes aiguës sont plus fréquentes chez l enfant. Les formes chroniques sont plus fréquentes dans le sexe féminin. La répartition entre les deux sexes est égale pour les autres formes.

Saison. La presque totalité des form deux périodes de l'année, hiver et prin autres formes est dispersée.

Début. Une thrombopénie chronique ment mais la soudaineté du début est p mes aiguës.

Clinique. Le syndrome de thrombop glantes buccales et hématurie, que nou très remarquable par sa ressemblance a Il n est pas spécifique. Les bulles sangl dant beaucoup plus fréquentes dans les formes chroniques. Ce fait mis à part, il n férences cliniques entre le purpura thron mière période d une forme chronique.

Hémostase. De même l étude de l h différence.

Etude isotopique. La durée de vie de séquestration sont les mêmes dans les d noté d influence de l ancienneté de la de vie des plaquettes.

Etude immuno-hématologique. L étude montre pas de différence nette. Tout au réactions immunologiques sont un peu n les purpuras aigus des enfants.

Ainsi, il est très difficile, sinon imp thrombopénie récente va évoluer sur u La fréquence plus grande chez les jeune en certaines saisons, la fréquence plus la fréquence plus grande des bulles bu grande des réponses immunologiques p des thrombopénies aiguës. Aucun de c formelle.

L évolution du nombre des plaq ques précisions utiles.

1 Si ce nombre des plaquettes n trois semaines, la guérison en un mois

2 Si le nombre des plaquettes n a p jour il faut s'attendre à une évolution ment six mois.

L'analyse de la fin des périodes thrombopéniques nous permet de relever les divers modes d'amélioration connus au cours des thrombopénies communes (évolution parallèle de la thrombopénie et des troubles de l'hémostase, évolution dissociée, état thrombopathique persistant quelque temps après correction de la thrombopénie).

Il s'agit d'un syndrome très remarquable, caractérisé par l'apparition irrégulière, à intervalles espacés, de périodes thrombopéniques et hémorragiques, l'évolution générale occupant plusieurs années. Nous n'avons pas observé de cas mortel. Nous n'oserions dans aucun cas affirmer la guérison.

Thrombopénies mortelles

Avec quelle fréquence, quand, comment, pourquoi meurent les sujets atteints de thrombopénie idiopathique? Notre étude permet d'ébaucher les réponses à ces questions fondamentales.

1° Nous avons déploré 10 morts sur 147 thrombopénies idiopathiques, soit 6,8 %. Le taux de mortalité de notre série est remarquablement proche de la mortalité générale (6,3 %). Cette constance des taux de mortalité mérite d'être soulignée.

2° Les morts *inopinées* dues généralement à une hémorragie des centres nerveux, interrompant soudainement le cours d'une thrombopénie paisible, sont depuis longtemps signalées. Elles sont probablement très peu fréquentes. Nous n'en avons observé aucun cas dans notre série.

3° Ce sont au contraire des morts *attendues et redoutées* que nous avons observées, évolution terminant des purpuras thrombopéniques inquiétants. Il nous paraît légitime ainsi d'isoler les *états thrombopéniques graves*. Ils ont été constatés exclusivement dans le sexe féminin, et avec une fréquence égale chez l'enfant et chez l'adulte (5/5) (tableau II).

Tableau II

	Cas féminins	Cas masculins
Thrombopénies en général	89	58
États thrombopéniques mortels	10	0

La gravité n'est pas immédiate mais tantôt c'est *rapidement* (avant 3 mois – 4 cas), tantôt c'est *après un an* (4 cas), tantôt et plus rarement c'est *après une longue période* (10 ans, 16 ans) que la thrombopénie revêt une forme grave.

Les *hémorragies* répétées, abondantes, de sièges divers, difficilement compensées par les transfusions, non taries par les thérapeutiques, composent un tableau alarmant, et à travers les variations individuelles, assez pareil d'un cas à un autre.

La mort est parfois due à une hémorragie cérébrale ou cérébroméningée. Elle est assez souvent la conséquence d'un accident des thérapeutiques, de cas *thérapeutiques* que la gravité de l'état conduit parfois à multiplier et pas toujours de façon coordonnée.

L'étude biologique ne reconnait pas de désordre constant et cohérant. Les faits suivants méritent pourtant d'être signalés. Le nombre des plaquettes (on sait les difficultés des numérations exactes) est souvent très bas et reste très bas. Le taux des mégacaryocytes médullaires (par définition normal ou supérieur à la normale au début) peut en cours d'évolution s'abaisser soit de façon passagère, soit progressivement. La présence à l'autopsie de cellules leucocytaires anormales (blastes?) est notée dans deux cas. Les études immuno-hématologiques isotopiques, l'étude de l'hémostase ne révèlent pas de désordres particuliers. Dans un cas, un anticoagulant circulant a été reconnu.

Il est difficile, en l'état actuel, de discerner si ces états thrombopéniques graves sont le fait d'une maladie particulière, ou s'ils sont l'expression commune de divers syndromes. Mais il est important de les reconnaître, de savoir qu'ils représentent la condition habituelle de l'évolution fatale, de tenter par une thérapeutique ordonnée de limiter la gravité de cette évolution.

Corticothérapie

Nous avons utilisé tantôt et le plus souvent la prednisone, tantôt d'autres cortico-stéroïdes dont l'action ne nous a pas paru différente de celle de la prednisone.

1 L'action qu'exerce la cortisone sur la thrombopénie est pour une part fonction de la dose. Mais la posologie efficace est moins forte que nous ne l'avions cru initialement. Les doses faibles (prednisone inférieure à 1 mg/kg/jour) ne modifient qu'inconstamment la thrombopénie. Les doses moyennes (Prednisone 1 à 1,5 mg/kg) et les doses fortes (Prednisone supérieure à 2 mg/kg) obtiennent dans une proportion comparable (et qui est élevée) la correction de la thrombopénie et le retour des plaquettes aux chiffres normaux. On notera que la remontée franche, supérieure à 200.000 plaquettes

par mm³ est plus souvent obtenue avec les doses fortes (13/15) qu'avec les doses moyennes (35/64)

L'étude isotopique montre que la corticothérapie agit lorsqu'elle est active, en réduisant la destruction plaquettaire excessive. Elle ne modifie pas sensiblement la production médullaire, ni dans le sens de l'augmentation, ni dans celui de la diminution, contrairement à ce qui a été soutenu. Nous avons dans un cas étudié à deux reprises la survie des propres plaquettes d'un sujet sous corticothérapie, d'abord complètement efficace, puis incomplètement efficace. Les courbes indiquent que la réapparition de la thrombopénie est due à une réapparition de l'hyperdestruction et non pas à une inhibition de la production médullaire. Inversement, et ce contraste doit être souligné, la corticothérapie ne modifie pas les réactions immunologiques qui restent positives même au cas d'amélioration induite par la corticothérapie.

2 Dans les formes aiguës le taux des plaquettes peut demeurer élevé après arrêt de la corticothérapie. Il est difficile d'interpréter ces faits et de choisir entre deux explications effet durable de la cortisone abrégeant la durée de la maladie ou plus vraisemblablement survenue au moment opportun de la guérison spontanée. En faveur de cette deuxième interprétation nous noterons l'absence de différence entre la durée des épisodes des purpuras aigus à rechute traités et non traités.

3 Dans les formes chroniques, l'action ne persiste généralement pas après arrêt de la corticothérapie. L'étude des courbes plaquettaires reconnaît une dose seuil de cortisone au-dessous de laquelle le nombre des plaquettes s'abaisse à nouveau. Dans un seul cas sur 79 malades traités la corticothérapie a obtenu une guérison définitive.

La cortisone n'est donc pas un traitement vrai des thrombopénies chroniques. Cependant la correction plaquettaire, même transitoire, même partielle peut être provisoirement utile. La corticothérapie doit être de courte durée. En cas d'échec après deux semaines de traitement, la prolongation de la corticothérapie n'est pas plus efficace et redoutable par l'hypercorticisme qu'elle entraîne.

Comment dès lors concevoir à l'heure actuelle *l'utilisation des corticoïdes* dans le traitement des purpuras thrombopéniques chroniques? L'indication varie selon qu'il s'agit d'un purpura à début récent, ou déjà passé à la chronicité, ou d'une phase hémorragique aiguë.

1. Si le malade est vu au début de l'affection, la corticothérapie doit être commencée immédiatement à doses modérées comprises entre 1 et 1,5 mg/kg de poids et par jour. La durée du traitement sera comprise entre trois semaines et un mois suivant l'évolution du chiffre plaquettaire à dose entière, puis à doses décroissantes pendant les 15 jours suivants. Aucun accident lié à la corticothérapie ne doit en principe survenir avec un tel traitement (en dehors des contre-indications classiques).

2. En cas d'échec ou de rechute rapide se posera le problème de l'indication des fortes doses. Deux conceptions sont possibles à cet égard : ou bien en cas d'échec complet après un délai de trois semaines, on augmente les doses initiales jusqu'à 2 à 3 mg/kg, ou bien la corticothérapie ne sera reprise à doses élevées qu'après un intervalle libre sans traitement. Aucun argument ne plaide actuellement pour l'une ou l'autre méthode. En cas de fortes doses la durée du traitement à dose entière sera limitée à 15 jours avec arrêt progressif en 15 jours.

3. Si l'affection est entrée dans la phase chronique, lorsque le malade est vu pour la première fois et en dépit de résultats négatifs obtenus jusqu'à maintenant, l'indication d'une corticothérapie de courte durée, à doses faibles, nous paraît cependant certaine, les délais et les modalités étant identiques à celles du purpura récent. Nos données actuelles sont en effet insuffisantes pour écarter la possibilité d'une guérison définitive. D'autre part, les résultats de la corticothérapie apportent des renseignements précieux pour l'indication de la splénectomie associés aux épreuves isotopiques pratiquées avant et sous traitement cortisonique. Le risque d'accidents d'hypercorticisme est limité au maximum par la brièveté du traitement.

4. Enfin la corticothérapie à doses élevées (2 mg/kg) reste le traitement des phases hémorragiques aiguës comme nous le verrons par ailleurs, soit qu'elle entraîne une remontée plaquettaire transitoire permettant l'arrêt des hémorragies, soit que, sans modifier le chiffre des plaquettes, elle corrige le retentissement sur l'hémostase (temps de saignement, résistance capillaire, coagulabilité globale).

Restent deux difficiles problèmes. Celui de la répétition des cures de corticothérapie. Nous avons observé chez un certain nombre de nos malades un épuisement de l'effet de la prednisone, la remontée plaquettaire ne se produisant plus pour une même dose lors de la reprise du traitement. Nous pensons personnellement

qu une reprise de la corticothérapie ne se justifie que par un accident hémorragique ou une modification des doses. Celui d'une dose liminaire continue faible constituant une sorte de seuil thérapeutique. Elle ne doit pas à notre avis être préférée à la splénectomie mais pourrait être envisagée en cas d échec secondaire de l intervention.

Splénectomie

Le beau mythe des effets immédiats et constants de la splénec tomie est doublement faux. La correction de la thrombopénie n est pas immédiate et surtout elle est inconstamment stable. Il n est pas possible de comparer utilement les statistiques apportées par des auteurs qui tantôt recommandent une splénectomie précoce et fré quente, tantôt attendent plusieurs mois, tantôt la réservent à quel ques cas exceptionnellement graves, exceptionnellement longs. Ceci d'autant plus que le même observateur instruit ou se croyant instruit par l'expérience, a modifié ses propres règles à plusieurs re prises et que chaque statistique personnelle est le plus souvent hété rogène. Mieux vaut se limiter à l'examen de problèmes précis.

Une première question est celle des indications des *splénectomies précoces* faites pendant les premiers mois, voire les premières semaines de la maladie. Deux motifs de splénectomie précoce, deux espoirs d'effet favorable doivent être discutés, espoir d'arrêter l'évolution fatale d une forme grave, espoir d'abréger la durée d une forme commune.

La situation pour les états thrombopéniques graves peut être décomposée de la façon suivante 1 La splénectomie, qui, en période hémorragique alarmante, est une intervention grave, ne peut être dans certains cas refusée lorsqu elle représente la dernière chance offerte au malade. Elle permet parfois de passer un cap dangereux. Elle n exerce le plus souvent au mieux qu une action très passagère. Notre expérience personnelle ici n est pas favorable. La splénectomie ne paraît pas constituer un traitement d urgence des thrombopénies hémorragiques. 2 La splénectomie pourrait dans ces formes graves être envisagée plus précocément, au moment où l'aggravation se produit. Nous n avons pas de documents personnels. 3 L évolution chronique d un purpura thrombopénique peut elle être entravée par une splénectomie précoce? La réponse à cette importante question ne pourrait être apportée que par une étude comparée faite par le même auteur de deux séries contemporaines

de thrombopénies traitées les unes par splénectomie, les autres sans splénectomie. Ce travail, à notre connaissance, n a pas été fait.

Il n est pas facile, moins malaisé cependant, de tenter de répondre aux questions posées pour les *splénectomies tardives*. Et d abord à celle-ci soit une thrombopénie ancienne datant de plus de six mois, de plus d un an. L'influence de la splénectomie est-elle vraiment heureuse? Cette question est surtout posée cliniquement lorsque la thrombopénie est paisible, ne s'accompagne pas d'hémorragies graves. La réponse ici peut être nette. 1 Le cours calme d'une thrombopénie peut, très tardivement parfois, se transformer et s'aggraver Notre étude des thrombopénies graves en témoigne. 2° Les chances de guérison des purpuras thrombopéniques sont beaucoup plus grandes pour les malades splénectomisés que pour ceux non soumis à l intervention.

Peut-on prévoir au moins approximativement, les effets heureux ou nuls de la splénectomie et, en conséquence, préciser les indications opératoires? Aucune réponse absolue ne peut être donnée ici, mais les remarques suivantes peuvent être faites.

1 L étude de la séquestration splénique, hépatique, ou mixte des plaquettes marquées au radio chrome apporte des informations utiles. Elle montre une corrélation certaine mais non parfaite entre le siège de séquestration splénique et l efficacité de la splénectomie. Cette démonstration de corrélation entre l efficacité du traitement et le siège de la destruction plaquettaire serait très importante.

Ajoutons que la survie des plaquettes chez les sujets splénectomisés a mis en évidence les faits suivants

a) La guérison d un purpura thrombopénique après splénectomie est due généralement à la suppression de l'hyperdestruction et non à une augmentation de production *durable*.

b) La thrombocytose initiale et parfois durable n est pas due à une vie des plaquettes supérieure à la normale mais seulement à une augmentation *passagère* de la production, deux ou trois fois la normale.

c) L arrêt de l hyperdestruction paraît très rapide. La vie des plaquettes (étrangères ou propres du sujet) est normale dès le cinquième jour d une splénectomie efficace. Nous n avons pas trouvé de fait suggérant une augmentation progressive de la durée de vie des plaquettes comme cela a été signalé par d autres observateurs.

d) Cependant, dans trois cas, la splénectomie a agi sur le taux des plaquettes étrangères sans augmenter sensiblement leur durée

de vie. Il s'agit de malades qui n ont été transfusés qu une fois au cours de l'intervention et, de ce fait, une iso-immunisation est difficile à admettre. Dans ces trois cas (sur 31) on devrait donc admettre que la splénectomie a été active avant tout par levée d une inhibition du fonctionnement médullaire.

Quelque soit le mécanisme par laquel la rate agit en physiologie ou en pathologie, l'étude isotopique a eu le mérite d'apporter deux données

– La splénectomie agit en restaurant une vie normale des plaquettes du malade ou des plaquettes étrangères normales (disparition de la destruction dans la rate ou disparition de la mise en circulation d un agent destructeur ou des deux mécanismes à la fois)

– La splénectomie agit en permettant à l'hyperplasie médullaire d être efficace (disparition d'un facteur destructeur du mégacaryocyte ou disparition d un facteur inhibiteur physiologique de la production médullaire ou abaissement de seuil d'aptitude à la mise en circulation normale)

2° *Immunologie*. La guérison spontanée est plus fréquente lorsque l'enquête immunologique est complètement négative.

On ne note pas de corrélation significative entre les résultats de l'étude immunologique et les effets de la splénectomie. Cependant le pourcentage des succès de la splénectomie paraît un peu plus grand pour les cas positifs que pour les cas négatifs.

Contrairement à la corticothérapie, la splénectomie a une action très nette sur le test de consommation de l antiglobuline qu elle négative complètement dans la moitié des cas. Cet effet, qui a peu de signification quend il est transitoire (répartition de l anticorps sur un beaucoup plus grande nombre de plaquettes) est très important quand il est définitif. Dans tous nos cas il accompagnait un succès définitif de la splénectomie.

Un succès apparemment total de la splénectomie peut cependant être observé alors que le test reste positif. Un recul insuffisant ne nous permet pas encore de savoir si de tels cas rechuteront.

3 L étude comparative des réactions à la corticothérapie et à la splénectomie apporte un argument non formel, à savoir la plus grande fréquence des guérisons chez les malades réagissant bien aux corticoïdes, mais il ne s'agit là que de fréquence relative.

4 Enfin, lorsque la splénectomie a été faite, le taux de l élévation plaquettaire post opératoire et aussi la durée de la période de forte thrombocytose ont une valeur pronostique à long terme.

L'étude de cette remontée plaquettaire permet les remarques suivantes. La remontée plaquettaire après splénectomie est variable. Elle dessine habituellement une courbe lente avec augmentation progressive du nombre plaquettaire. Le début de la remontée est précoce dès la 24ème heure après l'intervention. Le maximum est atteint à un moment variable compris entre le deuxième et le quinzième jour suivant les malades, habituellement autour du dixième jour. La durée de forte thrombocytose est également variable.

Lorsque le chiffre plaquettaire n'est pas remonté, dans les dix jours suivant la splénectomie, au-dessus de 100 000 on observe 100 % d'échecs dans un délai de deux ans après l'intervention. Par contre, lorsque le chiffre des plaquettes est monté au-dessus de 500.000, le pourcentage de guérison immédiate et définitive est élevé, 11 sur 19. Un seul échec vrai a été enregistré et le nombre de guérisons après deux ans est très significatif, soit 13 cas sur 16 malades suivis régulièrement. Les chiffres du pic plaquettaire post splénectomie nous paraissent dès lors prendre une haute valeur pronostique.

Essai de traitement par la 6-mercaptopurine

Les essais de traitement des thrombopénies idiopathiques par la 6-mercaptopurine sont inspirés par l'éventuelle origine immunologique de ces thrombopénies, par la connaissance de l'action qu'exerce la 6-mercaptopurine sur les cellules formatrices d'anticorps. En raison de l'inconvénient de l'emploi de la 6-mercaptopurine, ces essais ont été jusqu'à présent à des cas réservés rebelles à toute autre thérapeutique. La dose administrée est de 2,5 mg/kg/jour de 6-mercaptopurine pendant des périodes tantôt courtes, tantôt plus longues.

Nous avons ainsi traité quatre malades, trois après échec de la splénectomie, un avant splénectomie. Nous avons observé un échec complet, une remontée plaquettaire passagère et deux résultats durables. Dans l'un des cas la guérison se maintient depuis plus d'un an.

On trouvera la bibliographie complète dans «Semaine des Hôpitaux de Paris» (sous presse, 1964).

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The Hereditary Non-Spherocytic Haemolytic Anaemias

By J. V. Dacie

The hereditary haemolytic anaemias are a group of disorders of great interest and a good deal of information has recently become available as to the nature of the red-cell defects. In this talk I shall concentrate on the non-spherocytic types because of the recent advances that have been made in their understanding and also because as a group they are less well known than their companion disorders hereditary spherocytosis and hereditary elliptocytosis. In fact, the hereditary non-spherocytic haemolytic anaemias are not as uncommon as was at one time thought, and it is surprising that their recognition as a distinct group is a relatively recent event. Haden (4) in 1947 is generally accepted as being the first to suggest that a 'new' type of hereditary haemolytic anaemia existed. Not unexpectedly however with hindsight, it is possible to identify a few earlier reports in the literature of unusual cases of haemolytic anaemia as being of the non-spherocytic type, whilst in the last 10 years or so an increasing number of cases has been reported. Now it is known for certain, as had been suspected for a number of years, that we are dealing not with a single entity but with a group of disorders which differ both in pathogenesis and in their mode of inheritance.

Before dealing with the question of pathogenesis, I shall refer briefly to the main clinical and haematological features.

Anaemia is slight to severe there is acholuric jaundice and slight to moderate splenomegaly The diagnosis is usually made first in childhood, occasionally in infancy where the disease may simulate haemolytic disease of the newborn. The response to splenectomy is often negligible but it may be slight to moderate. The inheritance pattern is dominant or recessive in practice, the family history is often negative.

The anaemia is normocytic or macrocytic normochromic in type, and there is slight to moderate anisocytosis. There may be slight ovalocytosis but there is little or no spherocytosis, although an occasional contracted red cell with a slightly irregular outline may be seen. There is usually a moderate to high reticulocytosis. The blood films thus as a rule do not show impressive or characteristic abnormalities, but the films do, in different families, differ from each other in subtle ways some show more variation in red-cell size and shape than others, and in some cases punctate basophilia is quite conspicuous.

The osmotic fragility of fresh blood is within the normal range or there may be a slight increase in resistance, and there is a variable response to incubation. The rate of autohaemolysis (i. e. the amount of lysis occurring in sterile defibrinated blood incubated at 37 C for 48 hours) is generally increased but occasionally it is normal.

The osmotic fragility and autohaemolysis tests have received a good deal of attention and in 1954 a tentative classification was suggested (10) which divided cases into two groups, *Type I'* and *Type II'* Although this classification has been rendered more or less obsolete as the result of recent discoveries, it had, I think, a certain amount of value and it has in fact been quite widely used. The distinction between 'Types I and II' based on differences of behaviour in the osmotic fragility and autohaemolysis tests, is summarized in table I

Table I

The hereditary non-spherocytic haemolytic anaemias (after SELWYN AND DACIE, 1954).

Type	Osmotic Fragility		Autohaemolysis 48 hr, 37°C.	
	Before incubation	*After incubation 24 h, 37°C*	*Without added glucose*	*With added glucose*
'Type I	Normal	Increased but not more than normal. Fragility of some cells diminished	Normal or slightly increased	Diminished but usually less than normal amount
'Type II'	Normal	Greatly increased	Greatly increased (→ ×20 normal)	*Not* diminished by glucose

A striking finding was the observation that the addition of glucose had no effect in preventing haemolysis in the 'Type II' cases—as it does normally and as it does typically in hereditary spherocytosis. Moreover SELWYN and I (10) were able by actual measurements of glucose consumption to demonstrate that this was occurring in two 'Type-II' cases at only 25–30 / of the normal rate, if allowance was made for the high percentage of reticulocytes present. This work thus focussed attention on the possibility that a metabolic defect affecting the metabolism of glucose might be the basis for the rapid breakdown of the red cells of these patients both in vivo and in vitro.

Recent work has shown that cases diagnosed as 'Type II' on the criteria outlined above appear to be a definite entity while those classified as 'Type I' consist of several different disorders and incidentally included mild examples of 'Type II'.

Cases conforming to both 'Type I' and 'Type II' have been studied by DE GRUCHY and his associates (1, 2, 9) in Australia. First, DE GRUCHY et al. (2) showed that ATP (adenosine triphosphate, a key substance in the glycolytic cycle in the red cell), when added in vitro to the red cells of 'Type-II' cases, had a remarkable effect in diminishing the rapid rate of autohaemolysis although the addition of glucose failed to bring this about. This suggested that failure to synthesise sufficient ATP might be responsible for the red cells' impaired life-span. Next, ROBINSON et al. (9) studied the changes in concentration of the phosphate esters—important intermediates in the anaerobic metabolism of glucose via the Emden-Meyerhof pathway—when the red cells of their patients were stressed by incubation. In the 'Type II' cases there appeared to be a metabolic block beyond the synthesis of 2,3-diphosphoglyceric acid while in 'Type-I' cases the block appeared to be earlier than this (fig. 1).

More recently further advances have been made. In America, VALENTINE et al. (13) and TANAKA et al. (12) have shown that in cases conforming to the 'Type II' pattern the enzyme pyruvate kinase (PK) can be shown to be markedly deficient in activity. In Australia, too, DE GRUCHY (1) has found this same enzyme to be deficient in three out of four of his 'Type-II' cases and we in London have confirmed this in our two original 'Type-II' patients—the same patients who were investigated by SELWYN and myself in the early 1950's—as well as in some other cases subsequently studied.

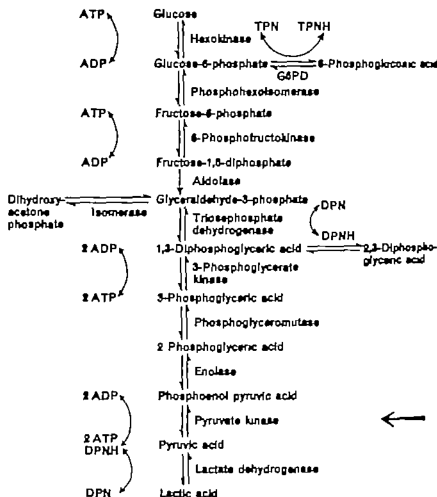


Fig 1 Embden-Meyerhof pathway of anaerobic glycolysis in human red cells. The position of the enzyme pyruvate kinase is marked by black arrow

Moreover VALENTINE and his co-workers (12 13) who reported on seven patients, obtained evidence by investigating their families that the disorder was inherited as a Mendelian recessive. They demonstrated, for instance, in one family that the pyruvate kinase activity of both parents of the propositus was lower than normal and in an extensive study that the enzyme activity in the presumed heterozygotes was about half the normal level, whereas in the patients themselves enzyme activity was almost absent. These observations explain the lack of evidence of direct transmission which is often found in patients suffering apparently from the 'Type-II'

disease. These interesting observations which have a bearing on the inheritance of the disease have been confirmed by other workers including ourselves.

Whether the enzyme is actually deficient in amount or whether it is present but has a reduced activity is uncertain. WALLER AND LÖHR (14) have suggested that the enzyme's affinity for its substrate phospho-enol pyruvic acid is reduced rather than that the enzyme itself is absent. The position of pyruvate kinase in the Emden-Meyerhof cycle is shown in fig 1

When the so-called 'Type-I' cases are considered the picture is less clear However some progress has been made in more than one direction. It has been shown that a small proportion of cases—with haematological characters bringing them into the 'Type-I' group—has a deficiency of the enzyme glucose-6-phosphate dehydrogenase (G6PD) Cases of this sort were first described in America by NEWTON AND BASS (6) in 1958 and ZINKHAM AND LENHARD (15) and SHAHIDI AND DIAMOND (11) in 1959 The patients have all been boys and the G6PD levels have been very low indeed.

Although the inheritance mechanism, with the abnormal gene being carried on the X sex chromosome, seems to be the same as in the enzyme deficiency which predisposes to drug idiosyncrasy haemolytic anaemia, the disease differs clinically from drug idiosyncrasy haemolytic anaemia in that haemolysis is continuous and in the laboratory in that the enzyme activity is very low even in newly formed reticulocytes. Moreover the disorder does not predominantly affect certain races, for example negroes, certain Jewish peoples and Mediterranean populations, as is the case in drug-idiosyncrasy haemolytic anaemia, for cases of chronic haemolytic anaemia associated with G6PD deficiency have been observed in England, Europe and Japan, as well as in America. The evidence suggests in fact that the disease depends upon a different mutant gene from that which brings about drug idiosyncrasy haemolytic anaemia.

However by no means all cases of 'Type-I' non spherocytic haemolytic anaemia are due to G6PD deficiency With my colleague Dr A. J GRIMES we have now looked for this deficiency in six families known not to have PK deficiency and in only two instances have we found deficiency of G6PD Several different types of abnormality are probably included in the remaining 'Type I' cases. From the genetical point of view the inheritance pattern in two

types at least points to the abnormal gene being inherited as a Mendelian dominant, with both sexes being affected. From the biochemical point of view the problem of pathogenesis remains so far largely unsolved.

De Gruchy (1) has reported low values of ATP which fell off rapidly when the blood was incubated, in three 'Type-I' cases (members of two families, all with normal pyruvate kinase) and our prototype 'Type-I' patient also has a low ATP value his PK activity is normal but there is, nevertheless apparently some impairment in the rate of red-cell glycolysis. Why the ATP level is so low in these cases is still a matter of speculation. De Gruchy (1) for instance, has, in his cases, studied the activities of all the enzymes in the Emden Meyerhof pathway and found them normal. He suggests as a possible explanation a defect in ATP production or its destruction.

Our own observations have demonstrated that one type of hereditary haemolytic anaemia which we had originally placed in the 'Type I' group on the basis of the usual laboratory tests is hereditary Heinz body anaemia—the patients I refer to had not had their spleens removed.

This rare disorder is inherited as a dominant and the characteristic Heinz bodies are not seen in the peripheral blood prior to splenectomy The pre-splenectomy blood films may however show an unusual degree of punctate basophilia and it is interesting to recall that punctate basophilia was a striking feature in one of Haden's (4) two original families. Now we know in hereditary Heinz body anaemia, as Grimes and Meisler (3) have recently demonstrated, that a significant proportion of these patient's haemoglobin is heat labile, being rapidly precipitated at 50 C. This disorder should therefore perhaps be looked upon as a haemoglobinopathy

Excluding our original 'Type I case, and a family with hereditary Heinz body anaemia, Dr Grimes and I have investigated two other patients in whom the results of routine haematological studies conformed to the 'Type I' criteria. The rate of red-cell glycolysis in these patients is not impaired, being commensurate with their reticulocyte count, and we have not been able so far to demonstrate the causal abnormality Both parents of one patient appear normal but the other belongs to a family where there is evidence of dominant inheritance.

I should add that PRANKERD (8) *has reported that he had ob*tained evidence of deficiency of the enzyme diphosphoglyceromutase in two cases (a father and son) and LÖHR AND WALLER (6) have more recently also reported indirect evidence in favour of deficiency of this enzyme in three unrelated patients. Furthermore, LÖHR AND WALLER (5) have reported deficiency of the enzyme glutathione reductase in three additional patients. As far as I know these are the only references to possible deficiencies in the activity of these enzymes. Finally I have to mention that several patients previously considered to be 'Type I' on the criteria put forward by SELWYN and myself (10) nine years ago have turned out to be mild examples of PK deficiency In fig 2 is summarized a classification of the hereditary non-spherocytic haemolytic anaemias which takes into account these recent advances in knowledge.

To return to the autohaemolysis test has this test at the present time any value in the differentiation of this group of anaemias or is it necessary to undertake the technically much more difficult en-

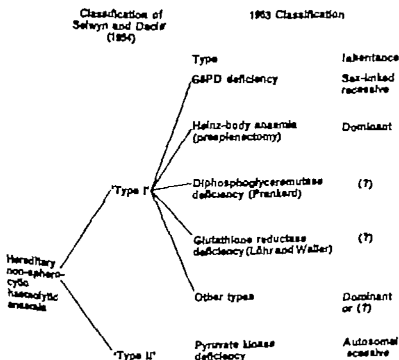


Fig. 2. Classification of hereditary non-spherocytic haemolytic anaemias.

Table II

Cases of hereditary non-spherocytic haemolytic anaemias. PGMS series: 1963.

Type I	G6PD deficiency	2 cases
	Heinz-body anaemia	4 cases (1 family)
	Not G6PD or PK deficiency	4 cases (3 families)
Type II	PK deficiency	9 patients (8 families)

zyme assays? With the assistance of Dr Grimes and Miss Leeth, I have been attempting recently in a number of patients with hereditary non-spherocytic haemolytic anaemia to relate the pattern of autohaemolysis and the effect of added glucose to what we now know of their red-cell defects. In all we have studied 19 patients, four of whom represent the original 'Type I' and 'Type II' cases studied by Selwyn and myself (10) The disorders from which they are suffering are given in table II. Our results can be summarized as follows

Starting with the nine patients who have PK deficiency autohaemolysis was increased beyond the normal range in all but two of them—who suffered from clinically mild haemolysis only In each case, however the addition of glucose to the incubating blood failed completely or almost completely to reduce the rate of lysis. This was true even of the two cases where the rate of haemolysis (without glucose) was within the normal range. The pattern of haemolysis and the effect of glucose was, on the other hand, less regular in the heterogeneous 'Type-I cases. In most, but not in all, lysis was slightly or moderately increased, but invariably the addition of glucose reduced lysis to some extent, although this might be proportionately less than with normal blood or with the blood of cases of hereditary spherocytosis.

In practice, therefore, the autohaemolysis test still seems to retain some usefulness, by giving a strong presumptive indication of PK deficiency if the addition of glucose to the blood fails to reduce the amount of lysis significantly it does not seem, on the other hand, to be of much help in separating the other types of case one from the other

It must not be forgotten, too that the autohaemolysis test is a non-specific one, and if failure of an increased rate of haemolysis to be reduced substantially by the addition of glucose is a reflection of an impaired glycolytic mechanism, which it seems likely to be,

then failure in reduction in haemolysis is likely to be found irrespective of the nature of the defect in glycolysis as long as this is severe enough. There seems no reason, therefore, why a marked deficiency of an enzyme other than pyruvate kinase could not result in the same degree of impairment of glycolysis and therefore in similar results in the autohaemolysis test.

One final point about the test. The results obtained depend to a considerable extent on details of technique, with strict cleanliness of glassware and an exact method of handling the blood and measurement of lysis being particularly important. I feel it is necessary for these reasons, for each laboratory where the test is carried out to set up its own normal standards.

Finally I should like to state clearly my view-point about the classification of the non-spherocytic haemolytic anaemias. The terms 'Type I' and 'Type II' introduced by SELWYN and myself (10) in 1954 have clearly outlived their usefulness and I am keen to discard them. As the abnormality affecting the prototypes of 'Type II' has been shown to be deficiency of the enzyme pyruvate kinase, there is no difficulty in dropping the term 'Type II' in favour of 'PK deficiency'. As some of the cases formerly classified as 'Type I' have now been shown to have G6PD deficiency or (rarely) to be examples of hereditary Heinz-body anaemia, there seems to be no great point in retaining the term 'Type I' either except perhaps as a temporary label for those cases, which are not too uncommon, where the red-cell defect still awaits clear definition in biochemical terms.

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Recent Studies in Autoimmunity*

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Ideas regarding autoimmunization date back to the pioneer days of immunology. It was early recognized by Bordet, Ehrlich and others that heteroimmune and isoimmune states could not only be discriminated but could be experimentally produced. When Donath and Landsteiner, Widal et al. and Chauffard and Troisier (1903–1914) demonstrated clinical evidence of autoimmunization in certain cases of hemolytic anemia, these studies received but little attention. It was not possible to induce autoimmunization experimentally and when Ehrlich introduced his now famous dictum of 'horror autotoxicus' it developed axiomatic quality and held sway for many years. To be sure, under normal circumstances, the body has certain regulatory devices which prevent its immunologic mechanism from destroying its own cells and tissues. On the other hand, as Dacie (1) has recently pointed out, the development of autoimmunization must be characteristic of an exceptional event, perhaps of a breakdown of the normal homeostatic mechanisms, or to put it more specifically as a possible breakdown in 'actively acquired immunologic tolerance'. However one looks at it, even the most skeptical must now agree that conditions exist, in both humans and animals, characterized by an abnormal immunologic response arising within the patient's own body as a result of which there is an attack on one or more of the patient's own cells or tissues.

Autoimmunity may be defined as (1) the development of abnormal immune antibodies (gamma globulins) (2) within the patient's own body with (3) these antibodies having the capability of reacting against constituents of the patient's own cells or tissues

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(and usually against those of other individuals) This definition does not necessarily state that autoimmunity and autoimmune disease are the one and the same there are, for example, immune antibodies which may circulate for years without any evidence of disease (the positive serology the rheumatoid factor the antithyroid antibodies, perhaps even the positive L. E. test in some individuals) Autoimmune disease is present when there are not only detectable abnormal immunologic constituents, but detectable abnormalities in the patient's cells and tissues. To be sure, clinical detection is not necessarily tantamount to actual clinical disease, but there must be some criterion for disease and this is the best one can do at this time.

Autohemolysins were found by DONATH AND LANDSTEINER (2) in paroxysmal cold hemoglobinuria and later by CHAUFFARD AND TROISIER (3) in cases of hemolytic ('hemolysinic') anemia. Autoagglutinins were discovered by the French workers, WIDAL, ABRAMI AND BRULÉ (4) in cases of acute hemolytic anemia and were related by these workers to the disease. Despite these important observations of 1908–1915 the whole matter of immunohemolysis and of autoimmune disorders in general was neglected for many years thereafter eventually to be revived in 1937 and subsequently and more recently to be snowballed into the ever increasing emphasis on autoimmune disorders.

With knowledge that an antibody is produced by an antigen, it was natural to ask of the clinical cases (1) where is the antigen? (2) how does one know that this material described as antibody is not simply a coincidental protein? (3) why cannot one reproduce autoimmune states in the experimental animal? Attempts to find the abnormal antigen were consistent only by their failure it was difficult to prove to the skeptical that the abnormal globulin was injurious to the cell or tissue in question and finally many attempts to alter the antigen of an animal and induce antibody formation in the same animal were unsuccessful. The whole emphasis was on the antigen it seemed altogether inconceivable that abnormal antibody could arise in any other way Actually several indications of antigen-oriented disease were eventually discriminated i. e., (1) long sequestred antigen becoming antigenic when suddenly liberated into the circulation—as in sympathetic ophthalmia (lens protein) (5) or in aspermatogenesis (sperm) (6) and in the HASHIMOTO thyroiditis (7) In the latter disorder as so well demonstrated

WITEBSKY et al., there was experimental evidence for the possibility that thyroglobulin, long sequestered in thyroid and liberated into the circulation, might become antigenic. This material like lens protein and spermatozoa, could be conceived of as having been (a) produced late in fetal life, leading (b) to the presence in certain sequestered areas of materials unknown to the body's immunologic apparatus (c) such materials, when liberated into the circulation, would be unrecognized and thus be antigenic (d) this would result in the formation of abnormal antibody

Hapten-mediated disorders such as quinidine or sedormid thrombocytopenic purpura represented a second form of antigen mediated autoimmunization. Hapten mediation, in the phenomenon of immunization, was one of LANDSTEINER's chief works (8) which he carried out in a series of brilliantly designed animal investigations. We first used this concept in the attempt to explain aminopyrine agranulocytosis (9) and later sedormid and quinidine thrombocytopenia (10) It seemed quite likely that (a) the combination of drug and either white cell or platelet caused modification of the cell which now became antigenic, (b) successive administrations of drug resulted in increasing antigenicity and the increasing development of specific antibody (c) when sufficient antibody was present and the drug was again given, this would result in leukopenia or thrombocytopenia, as the case might be. In sedormid thrombocytopenia, as ACKROYD pointed out (11) the serum containing antibody alone could not induce thrombocytopenia, but the serum plus the drug and the platelets resulted in severe reduction of platelets.

More recently this interpretation has been placed in question it is now considered likely that the chemical or drug is itself antigenic and that the platelet is used simply as a reacting surface or staging area (12) According to this concept, the antibody is directed specifically against the chemical. The combination of chemical antigen with specific antibody (antigen antibody complex) is absorbed by or reacts with the platelets, which are then altered and quickly destroyed, with resultant thrombocytopenia.

These two concepts of antigen-oriented autoimmune disease kept alive the idea of antigen as an important factor in autoimmune disease. The first one—that of sequestration seemed logical and was bolstered by experimental evidence. The second, that of the hapten mechanism, has become dubious, at least with reference to

clinical disease. Furthermore, attempts to modify antigen, to fortify it with Freund's adjuvant, etc., were unsuccessful in inducing autoimmune hemolytic disease, ITP or leukopenic states, although disturbances of organs could be so induced. The abnormal antigen as an etiologic factor in autoimmune disease did not appear adequate to explain such autoimmune disorders as autoimmune hemolytic anemia, systemic lupus, etc. This eventually led us to the concept that perhaps it was not antigen, but rather the abnormal antibody tissue, which could produce abnormal antibodies in response to the normal antigens present in the body

The idea that abnormal antibody producing tissue might be responsible for the development of abnormal autoantibodies arose from our observations in chronic lymphocytic leukemia, generalized lymphosarcomatosis and infectious mononucleosis (13 14) In these lymphoproliferative disorders, whether benign or neoplastic, immunologic abnormalities were common. Could it be that the lymphoid proliferation, composed as it was of immunocompetent cells (immunocytes) was foreign or abnormal to the body? If so normal red cell antigens, for example, could come to the foreign tissue and fail to be recognized as self" thus inducing the production of abnormal autoantibodies. This seemed likely for infectious mononucleosis where the highly abnormal cells might even be responsible for the production of the highly abnormal heteroagglutinins. In chronic lymphocytic disease, the fever wasting and cachexia of its latter stages, could possibly be explained as a graft (the leukemic tissue) reacting against the host' (the patient), as in experimentally induced homologous or runt disease.

Runt disease is a by product of the considerable interest in recent years of the transplantation phenomenon. It may be induced in various ways, but fundamentally it requires (a) the introduction of foreign immunocompetent cells which are (b) tolerated by the host. This combination leads to reaction of the grafted cells against the host, the normal host antigens having stimulated the production of abnormal antibodies reacting against the host antigens. In the work of OLINER AND SCHWARTZ (15) in our laboratory in which immunologically competent spleen cells (lymphocytes) from one of the parents were injected into F_1 hybrids, it was shown that autoimmune hemolytic anemia, thrombocytopenic purpura, leukopenia, alopecia and wasting were produced regularly in this homologous syndrome. This condition had many of the features of the auto-

immune disorders, although in actuality it was isoimmune (homologous, in transplantation parlance) since foreign cells from a genetically dissimilar animal had been injected. On the other hand it could be stated that the conditions were quite close to the autoimmune state since the foreign cells from the parent were tolerated by the host in this tolerant state the grafted cells then reacted against the host with the resultant features of this interesting syndrome. The reaction was one in which (a) the normal host antigens, coming (b) to the abnormal or foreign immunocompetent cells, stimulated (c) the production of abnormal antibodies which reacted (d) against the normal antigens thus causing either hemolytic anemia, thrombocytopenia and other abnormalities, including the alopecia and the wasting

These animal experiments gave us considerable insight into the clinical phenomena of autoimmunity as seen in the lymphoproliferative disorders and in systemic lupus. In chronic lymphocytic leukemia, for example, one might consider that the leukemic tissue was a graft' of abnormal, but immunologically competent cells within the host (15) This graft apparently lived in tolerance with the 'host' patient, often for many years, but this tolerant state could be broken at times, leading to various immunologic phenomena—chiefly AIHA, at times, ITP at times, other conditions. The ITP-like disorder of chronic lymphocytic leukemia has only recently received some attention. It occurs either with or without AIHA and appears to represent an autoimmune process in this disease, albeit not as common as hemolytic anemia. Other immunologic or presumed abnormalities that we have noted in the lymphoproliferative disorders include the following

AIHA	+ serology'
ITP	Heterophile agglutinins
Rheumatoid arthritis	Macroglobulinemias
Mikulicz syndrome	Cold hemagglutinin disease
Henoch-Schönlein vascular purpura	
Systemic lupus	Wasting

These various abnormalities could be the result of the activity of abnormal but immunologically competent tissues reacting against the normal host antigens. Either 'warm antibodies or cold reacting antibodies could be found the latter were chiefly macroglobulins

and of them, one was the cold hemagglutinin. The relationships of cold hemagglutinin disease to macroglobulinemia and chronic lymphocytic leukemia have proved of unusual interest and it seems clear that these apparently different disorders are, at least in some instances, closely related. One begins to wonder in some of these cases, whether the lymphocytosis present is truly leukemia or perhaps the cellular indication of an immunoproliferative reaction. The gray area between leukemia and the immunoproliferative response is no better illustrated than in so-called macroglobulinemia.

In systemic lupus, one can speculate that the body has developed abnormal immunologic techniques which eventually result in its own destruction. At the beginning there may be only a positive serologic test for syphilis (biologically false positive) later rheumatoid arthritis or autoimmune hemolytic anemia may develop, finally the whole gamut of the disease with renal involvement, skin rash and a positive L. E. test become apparent. This may take one year it may take twenty (16) It is conceivable that the condition is, in some instances at least, genetically determined, perhaps through the medium of inherited, genetically determined abnormal clones of immunocompetent cells. It is conceivable that these may show varying degrees of activity at some time in the patient's course. The final process of wasting, alopecia and the numerous manifestations of autoimmune disease (hemolytic anemia, leukopenia, thrombocytopenia) seem analogous to the findings in runt disease, in which abnormal immunocompetent cells have been introduced into a normal recipient.

There can be no question but that the status of the proliferating cell in the immune response has become increasingly important. As a simplification, we can say that there are at least two kinds of immunologically competent cells (immunocytes) the lymphocytes and the plasmocytes. There is abundant evidence that the plasmocytes produce various types of immunoglobulins, chiefly of the 7S variety (gamma$_2$, gamma$_{1A}$, beta$_{2A}$) and the microglobulin known as the Bence Jones protein. Evidence that the lymphocytes produce immunoglobulins is not quite as clear but from such experiments of nature as macroglobulinemia it seems that lymphocytes, whether typical or reticular' produce macroglobulins. The immunocytes are to be considered as those cells concerned in the immunoproliferative response which may include as well the reticulum cell although the place of the latter cell and indeed

of the 'reticuloendothelial system ist not clear in this context. Doubtless, such phagocytic cells may capture antigen, but whether they then evolve into immunocytes is not certain, especially since recent studies have indicated the revolutionary concept that the apparently end-stage mature lymphocyte may under appropriate antigenic stimulation, evolve into a large, basophilic, pyroninophilic, primitive cell called, among other terms, hemocytoblast or immunoblast (17) A going hypothesis at this time is that the lymphocytes (or *some* lymphocytes) will become transformed by antigen into immunoblasts these in turn, may produce either lymphocytes of plasmocytes, perhaps dependent upon the type of antibody stimulation.

At least two types of immune reaction have been discriminated that of *humoral antibody production* which appears to be largely plasmocytic in origin and concerned chiefly with 7S gamma$_2$ globulin and that of *cellular hypersensitivity* in which the proliferating cell seems predominantly to be the lymphocyte. The homograft rejection phenomenon is thought to represent, at least in large measure, a reaction of cellular hypersensitivity in which it is difficult to detect humoral antibody As time goes on, the differences between the two types of immunity—humoral and cellular—may well become blurred. In any event, the fundamental event in the immune response is the cellular reaction. Whether this occurs through the mediation of previously determined groups or clones of immunocompetent cells (clonal selection) or by an instructive method, in which uncommitted lymphocytes react for the first time with antigen is a matter largely of opinion. Logic seems to be on the side of the instructive hypothesis, although undoubtedly committed groups of cells may be present, either because of previous contact with the same antigen or through inherited or other mechanisms.

For purposes of the discussion here, the 'primary response may be said to represent that of uncommitted, immunocompetent cells the secondary or anamnestic response that of committed cell groups (clones) These may be scattered throughout the body and ready to react with an antigen previously present or these committed clones may conceivably be abnormal (forbidden) (18) but non-reactive or partially reactive, because they are tolerated by their host. It is conceivable that such forbidden clones may have been inherited from the parent or that some of the mother's im-

the fetus, with resulting tolerance (19) It also is possible that mutational activity induced by ionizing radiation, chemicals, and viruses may have resulted in the development of such abnormal clones which in some manner are tolerated by the host patient.

The idea of an abnormal immunologic complex or an inherited forbidden clone has been advanced by the findings of a high incidence of immunologic disease in first and second degree relatives of individuals with systemic lupus, rheumatoid arthritis, autoimmune hemolytic anemia and other autoimmune disorders (20) Even more important are the observations in the New Zealand black mice with genetically determined autoimmune hemolytic anemia (21) and in chocolate brown New Zealand mice showing all the features of systemic lupus (22)

The concept of the abnormal, but tolerated clone of immunocompetent cells, would help to explain the presence of positive serologic test for syphilis or of the L. E. factor or of the rheumatoid factor in certain individuals free of any other indications of disease. Such individuals may develop—as we have seen—well-defined indications of systemic lupus or rheumatoid arthritis at some time in the future. In lupus this may be brought about by a gross exposure to ultra violet radiation. Similarly individuals with abnormal groups of lymphoid cells, e. g chronic lymphocytic leukemia may be entirely asymptomatic, but when given X-ray therapy or an alkylating agent, may then develop autoimmune hemolytic anemia or ITP or some other immunologic abnormality Such abnormalities may occur spontaneously' but only after 5–15 years of asymptomatic disease, at which time febrile episodes, weight loss and cachexia bring the patient to the termination of his disease. These various manifestations may indicate that (a) there is a reaction of the grafted abnormal but immunocompetent cells against the host patient, (b) that the previous state of tolerance between 'host' and graft has been disrupted—perhaps by such trigger' mechanisms as ionizing radiation, chlorambucil and the like. Such an apparent breakdown in tolerance has recently been demonstrated by Schwartz in our laboratory (23) in F_1 hybrid mice which survived the runted state induced by the injection of parental spleen cells, but when given a small dose of radiation developed rapidly all the various manifestations of a complex autoimmune disorder Kaplan and Smithers (24) alluded to a similar clinical possibility some years ago.

Further studies of the New Zealand black mice with autoim mune hemolytic anemia led BURNET to the thymus (25) Here he found a distinct abnormality the presence of germinal follicles in the framework of the normal lymphoid proliferation. Did these follicles, which were distinctly abnormal for the thymus, represent abnormal, functioning forbidden clones which were being disseminated by the thymus to other structures? This finding, together with other data, led BURNET to postulate that the normal thymus was the seat for uncommitted clones of lymphocytes, that when these become peripheralized in spleen or lymph nodes, they might then become committed in response to antigen. To some extent this represented a step back from BURNET's theory of clonal selection.

Work on the thymus was now proceeding rapidly apace, and its relationship to autoimmunity was being discussed in several quar ters. It was now apparent, as HEWSON had demonstrated more than 200 years ago (26) that the thymus was the primordial side for the proliferation of lymphoid cells. The studies of ADNER AND SHERMAN (27) in our laboratory have indicated that the fetus and the very young hamster's thymus is composed almost entirely of large, pri mitive appearing cells having the appearance of immunoblasts presumably these become peripheralized to lymph nodes and spleen. Without the thymus, this spread of lymphocytes cannot occur and as a result, there is little or no lymphoid growth, im munologic competency diminishes and wasting occurs. What the thymus does in a more active way has been speculated upon by BURNET (25) There is some evidence that autoimmune hemolytic anemia in NZB mice may be based on a thymic lesion, in which abnormal clones of already committed immunocytes are dissemin- ated, later to result in disease (21 a) The findings of similar changes in myasthenia gravis, which looms quite large as a probably autoimmune disease (28) and in some cases of systemic lupus are in favor of this hypothesis (29) Preliminary work in our laboratory indicates that direct thymic stimulation by antigen may induce thymic lesions and certain indications of autoimmune disease (30) At any rate, the whole of the thymus story has not yet been told.

Finally in this brief and rather personalized survey of recent studies in autoimmunity we come to the matter of therapy Ini- tially we depended heavily upon splenectomy as the chief thera peutic agent in autoimmune hemolytic anemia and ITP (31) Later as it became increasingly evident that this operation was often un

successful and that antibody formation continued, we turned to agents to suppress or control antibody production. In 1935 when the reticuloendothelial system seemd to be the prime site of antibody production, we used Congo red in the attempt to block it, but without success (32) In 1945 following our introduction of nitrogen mustard for the treatment of Hodgkin s disease and lymphosarcoma, we used this agent—again to suppress the reticuloendothelial system (32) There were occasional successes. However when sufficient supplies of ACTH and then of the corticosteroids became available, we turned to these agents (33) because they had already been shown by WHITE AND DOUGHERTY (34) to cause reduction in lymph node and splenic size, and to result in lymphocytolysis and reduction in antibody production. The results with the use of adequate amounts of these agents in AIHA (33) and later in ITP (35) were striking and, indeed, led to a revolution in the therapy of the various autoimmune states. However as everyone knows, they are not without their side effects, particularly when continued maintenance therapy is required so that splenectomy is still essential in some cases (perhaps 2–3 of 10 in contrast with 8 of 10 previously) In addition, the experiments of SCHWARTZ and myself using the antimetabolic, 6-mercaptopurine, indicated that this agent was a potent suppressor of immunity in the experimental animal (humoral antibody delayed hypersensitivity homograft rejection, development of immunologic tolerance) (36a, b c) In studying the histology of the draining lymph node adjacent to the skin graft (ARNAT et al. 37) the striking proliferation of immunoblasts and lymphocytes resembling acute leukemia or primitive lymphosarcoma was evident. With the use of 6-MP and a take of graft accomplished, there was a disappearance of immunoblasts and of immature lymphocytes. When these experimental results were carried over into the clinic, it became evident that the drug was highly effective in most cases of AIHA, and could thus be used as a second or alternative agent to the corticosteroids when these were either ineffective or had resulted in reactions (38) Results in failed cases of ITP are under study The results in systemic lupus are not nearly as good as with AIHA to some extent, this is due to the frequency of gastrointestinal reactions requiring discontinuence of drug Good results have been obtained in such conditions as chronic Henoch-Schönlein vascular purpura and in dermatomyositis. In any event, here one has an agent or a class of agents, the antileukemic drugs, which may

be used as alternative therapy to the corticosteroids in the management of the autoimmune disorders and as a means of avoiding splenectomy or as a supplement to that operation, when it has been proved unsuccessful. Finally it should always be realized that the autoimmune disorders are fundamentally medical in their aspects and that the surgical removal of the spleen may do but little to control production of antibodies by the generalized immunocompetent tissues. Whether thymectomy will have a place in this general area of autoimmune disease remains to be seen.

In summary we can say that there have been many conceptual advances in the last few years in the consideration of the autoimmune disorders. These may be considered as exceptional events, in which a protective mechanism—the immune system—is turned against the tissues it is supposed to protect. This must indicate that the self recognition mechanism of immunocytes vis-a-vis, e. g. blood cells, among others, is at fault. We would like to believe, at this point, that this is due to the presence of groups or clones of abnormal immunocompetent cells which fail to recognize normal cell antigens and thus produce antibodies—autoantibodies—against them. Our concepts regarding autoimmunity have thus centered about the immunocompetent cell (immunocyte) immunocyte proliferation and its relationship to leukemia, the thymus as the important primordial site for lymphoid and immunologic competence, the graft vs. host phenomenon with particular respect to the lymphoproliferative disorders and systemic lupus, the abnormal or forbidden clone, the state of broken tolerance, etc. Such concepts, whether entirely true or not, have proved useful in our understanding of the pathogenesis of the autoimmune disorders, their development and course and finally their treatment.

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Porphyries cutanées tardives apparues à la suite d'affections hématologiques

Par A. GAJDOS

L'étiologie génétique des porphyries a été, jusqu'à ces derniers temps universellement admise. On a attribué ces affections, suivant la conception de GARROD à un «inborn error» du métabolisme. Cette manière de voir n'a plus une validité générale. Tout au moins, pour la porphyrie cutanée tardive, WALDENSTRÖM a complété en 1957 sa classification ancienne en admettant une forme symptomatique (1). On a envisagé en effet, d'ailleurs sans preuve absolue, que l'éthylisme chronique et la sous-alimentation sévère peuvent constituer des facteurs étiologiques.

La réalité des porphyries cutanées *acquises* est devenue indiscutable à la suite de l'observation récente de plusieurs milliers de cas en Turquie déterminés par une intoxication collective par l'hexachlorobenzène (2). Le rôle étiologique de cette substance a été établi avec certitude par la disparition de la maladie dans le territoire turc après la suppression de l'apport toxique et par la production d'une porphyrie expérimentale à la suite de l'administration du toxique aux animaux de laboratoire (3, 4).

Nous avons récemment observé cinq cas de porphyrie cutanée survenus chez des sujets atteints d'autres affections, elles-mêmes relativement rares. Dans deux de ces cas (5, 6) la porphyrie a été précédée de peu respectivement par un anévrysme de l'aorte et par une tumeur maligne du thymus. Dans les trois autres cas, la porphyrie est apparue chez des sujets atteints d'une maladie hématologique. Ce sont ces trois observations qui font l'objet de cet exposé.

Observations Cliniques

I. Dans la *première observation* publiée avec M. SELIGMAN, P. MEYER ET J. BERNARD (7), les premières manifestations d'une porphyrie cutanée ont été remarquées trois ans après le début apparent d'une macroglobulinémie de WALDENSTRÖM.

M. Per… âgé de 40 ans est hospitalisé en Mars 1960 pour asthénie, purpura des membres inférieurs, gingivorragie et épistaxis notés depuis 1958. A l'examen, on constate la présence d'importantes adénopathies cervicales et axillaires bilatérales, une splénomégalie franche et une hépatomégalie modérée. Le myélogramme montre l'infiltration de la moelle osseuse par des cellules lymphocytaires et plasmocytaires. La vitesse de sédimentation est très accélérée (105 mm en une heure) et la protidémie atteint 99 g par litre. Le diagnostic de macroglobulinémie de Waldenström est confirmé par l'ultracentrifugation mettant en évidence une augmentation importante du taux plasmatique de la bêta-macroglobuline et par l'examen histologique des biopsies médullaires et ganglionnaires.

Malgré l'administration de divers médicaments, l'évolution est marquée, au cours des années 1960–1961 par la fréquence des hémorragies (surtout épistaxis) entraînant une anémie importante, par une ostéoporose rachidienne très douloureuse et par une asthénie importante.

Pendant l'été 1961 plusieurs poussées d'éruptions bulleuses apparaissent au niveau du dos des mains et laissent des cicatrices pigmentées. On note également une pigmentation modérée des régions temporo-maxillaires et l'émission d'urines rouges. Le diagnostic de porphyrie cutanée est confirmé par l'analyse urinaire qui donne les résultats suivants: porphobilinogène = 2,5 mg/24 heures, uroporphyrine = 0,837 mg/24 heures, coproporphyrine = 1,230 mg/24 heures. La chromatographie sur papier et la détermination du point de fusion des cristaux de l'ester méthylique de l'uroporphyrine isolée des urines permettent d'établir que le pigment est constitué surtout par l'isomère III. Nous retiendrons d'autre part, que le malade nie énergiquement tout abus d'alcool.

L administration d'acide adénosine 5-monophosphorique (AMP) que nous avons préconisé pour le traitement des porphyries (8) ne donne que des résultats médiocres et incertains.

Ce n'est qu après plusieurs mois de traitement que nous avons observé une diminution de la photosensibilité et la baisse du taux urinaire de l'uroporphyrine qui tombe à la valeur de 0,415 par 24 heures en Mars, et de 0,320 mg en Mai 1961 Au printemps 1962, le malade étant toujours sous traitement par AMP aucune éruption nouvelle apparaît. Par ailleurs, les signes cliniques de la macroglobulinémie (asthénie, hémorragies) persistent invariables.

En résumé, chez un sujet de 40 ans atteint d'une macroglobulinémie, apparaît une porphyrie cutanée absolument typique, dont l'évolution est à peine influencée par l'administration d'AMP

2. Dans notre *deuxième observation*, rapportée avec J Bousser, D Christol, M. Gajdos-Török et A. Netter (9), *la porphyrie cutanée est précédée par une longue et complexe histoire hématologique caractérisée essentiellement par une anémie hémolytique grave à anticorps.*

Mme D… est hospitalisée en 1953, à l âge de 38 ans pour une anémie extrêmement grave globules rouges 840.000, plaquettes 90.000, globules blancs 4.200 dont 70% de polynucléaires neutrophiles. Sous l'influence de transfusions, l'anémie se répare progressivement. Deux mois plus tard, une rechute sévère nécessite une nouvelle hospitalisation. L anémie est importante (2.200.000 globules rouges). La moelle osseuse, riche en éléments cellulaires, ne renferme que 4,5% d érythroblastes. Le test de Coombs direct est positif avec agglutination des hématies trypsinées. Le diagnostic d'aplasie médullaire cryptogénétique est porté. Contrairement à toute attente, l'anémie se répare en un mois sous l'effet de transfusions sanguines. Le nombre des plaquettes s'élève à 150.000 et la proportion des érythroblastes dans la moelle sternale atteint 24,5%.

Mais une nouvelle hospitalisation est nécessaire en avril 1954 pour fièvre à 40° C, céphalées, épistaxis, arthralgies et myalgies généralisées. Quelques jours après l'admission, alors que les signes fonctionnels s'aggravent, apparaît un syndrome hémolytique sévère. Les recherches immunohématologiques décèlent l'existence d'agglutinines actives contre les trois lignées des éléments cellulaires du sang. Après une amélioration transi-

toire, obtenue par des transfusions sanguines et l'administration de cortisone, l'anémie aggrave de nouveau. On se décide, en Juin 1954 à une splénectomie, dont l'effet sur l'anémie hémolytique est favorable.

En Juillet 1953 un syndrome abdominal douloureux aigu, avec vomissements incoercibles et déshydratation importante, nécessite une nouvelle intervention chirurgicale. Elle permet de déceler une sténose médiogastrique d origine adhérentielle. Huit jours après l opération surviennent des hématémèses importantes. Le nombre des plaquettes tombe à 100.000. Après de nombreuses transfusions sanguines, la malade se rétablit lentement.

Entre 1955 et 1961 la malade consulte fréquemment pour une anémie modérée, des troubles dysménorrhéiques et un état dépressif.

C'est en 1961 qu pparaissent les signes d'une porphyrie cutanée: pigmentation des téguments et hypertrichose localisée surtout au visage, éruptions bulleuses au niveau des mains. L analyse urinaire confirme le diagnostic. Les urines de 24 heures renferment en effet 3,7 mg de porphobilinogène, 1,66 mg d uroporphyrine (dont l'isomérie III est établie par la suite) et 0,480 mg de coproporphyrine. On peut écarter avec certitude une éventuelle origine éthylique. D'autre part l'enquête familiale reste négative.

Le traitement par l'AMP diminue à plusieurs reprises l excrétion urinaire de l'uroporphyrine et améliore les signes cutanés.

3. Notre *troisième observation* est encore plus complexe que les deux autres. *La porphyrie cutanée apparue à l'âge de 7 ans, est en effet précédée par une série de divers états pathologiques: infections multiples, hyperleucocytose sanguine avec hyperlymphocytose permanente, purpura thrombopénique. Ce tableau est compliqué encore à l'âge de 10 ans d'une anémie hémolytique à rechute* (10).

L enfant Bernard P né le 2 Décembre 1950 est atteint de nombreuses infections. A l'âge de 2 mois otite, puis infections des voies aériennes, pneumonie, quatre épisodes de méningite suppurée à pneumocoques. Aucun trouble biologique est trouvé permettant d'expliquer cette fréquence des infections. Notamment l immunoélectrophorèse, pratiquée à quatre reprises, montre toujours un taux élevé de la gamma-globulinémie. L évolution des anticorps après les diverses vaccinations est révélée normale.

L hyperleucocytose et la lymphocytose, découvertes à l'âge de 2 ans, persistent jusqu aux derniers examens. Elles atteignent parfois des valeurs très élevées. On compte par exemple, au mois de Septembre 1961 150.000 globules blancs par mm³ dont 82% de lymphocytes. Cette leucocytose n est accompagnée ni d adénopathie, ni de modifications notables du myélogramme.

Le purpura thrombopénique débute à l'âge de 5 ans, se complique dans les deux ans suivants par trois poussées d'hémorragies cutanées et muqueuses, dont l'une est très inquiétante par une hémorragie méningée. Le nombre des plaquettes oscille entre 10.000 et 80.000. La recherche d anticorps antiplaquettaires est négative à plusieurs reprises. Une splénectomie est pratiquée à l'âge de 6 ans, qui semble d'abord guérir le purpura, mais 2 ans plus tard, en 1960, survient une nouvelle poussée de purpura thrombopénique.

Une porphyrie cutanée absolument typique apparaît à l âge de 7 ans, avec éruptions bulleuses aux parties découvertes de la peau, pigmentation cutanée, hypertrichose, coloration rouge des urines. Les urines de 24 heures renferment une quantité normale de porphobilinogène (2,5 mg) 1 4 mg d'uroporphyrine (appartenant à l'isomère III) et 0,123 mg de coproporphyrine. L uroporphyrine est également identifiée dans le sérum sanguin et les matières fécales. L uroporphyrinurie, dosée à de nombreuses reprises pendant les trois années suivantes, oscille entre 0,017 mg et 1 7 mg par jour.

Malgré le jeune âge du malade, on peut écarter le diagnostic de la porphyrie congénitale. On observe en effet ni l'érythrodontie, ni la fluorescence rouge des érythroblastes médullaires en lumière ultraviolette, signes constants de la maladie de Günther.

Le tableau clinique se complique encore à l'âge de 10 ans par une anémie hémolytique, dont la première manifestation, au mois de Mars 1961 est relativement lé-

gère et dure trois semaines. Un deuxième épisode hémolytique, beaucoup plus sévère, survient cinq mois plus tard, provoquant une chute du nombre des hématies à un million par mm³. On ne constate ni la présence d'une hémoglobine anormale, ni une déficience enzymatique des globules rouges. La résistance osmotique est normale.

L'AMP est administré d'une façon prolongée. Aucune amélioration objective de la porphyrie n'est observée.

Discussion

Dans les trois cas que nous venons d'exposer la porphyrie cutanée est apparue à la suite d'affections hématologiques rares et complexes. On peut exclure le rapport inverse, selon lequel les manifestations hématologiques constitueraient des complications d'une porphyrie cutanée méconnue. La seule forme des porphyries présentant des incidences hématologiques et notamment une anémie hémolytique, est en effet la maladie de Günther dont nous pouvons écarter avec certitude le diagnostic chez nos trois malades.

On peut se demander d'autre part, s'il ne s'agit pas, dans nos observations, de simples coïncidences. Nous ne le croyons pas. Ces trois cas, relevés au cours des trois dernières années, représentent 18 % des 17 cas de porphyrie cutanée que nous avons diagnostiqués pendant la même période. S'agissant des maladies rares, ce pourcentage nous paraît trop élevé pour admettre des coïncidences fortuites.

Par quel mécanisme les affections hématologiques, dont étaient porteurs nos malades, pouvaient-elles favoriser l'apparition d'une porphyrie cutanée? Nous sommes incapables de répondre à cette question, même par une hypothèse de travail. Nous voudrions seulement remarquer que la pathogénèse des porphyries en général reste à l'heure actuelle tout à fait mystérieuse. L'obtention des porphyrines et de leurs précurseurs physiologiques (acide delta-aminolévulinique et porphobilinogène) à l'état chimiquement pur a permis d'établir que ces substances sont dépourvues d'actions toxiques ou pharmacodynamiques. Leur accumulation dans l'organisme ne peut donc être responsable du déterminisme du tableau clinique des porphyries. On admet actuellement qu'un autre facteur de nature sans doute biochimique, doit être en jeu. Mais on ne sait rien encore de la nature de ce principe – le facteur X de GOLDBERG et RIMINGTON (11) – dont la production dans l'organisme peut être en rapport avec la biosynthèse exagérée des porphyrines, mais qui peut également en être la cause.

Nous croyons que c'est précisément notre ignorance complète à ce sujet qui confère un intérêt réel à nos observations. La reconnaissance d'autres cas semblables et leur étude minutieuse pourront peut-être contribuer au progrès qui reste à réaliser dans le domaine clinique des porphyries.

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The X linked Blood Group System Xg

Work in Progress

By R. R. Race and Ruth Sanger

Until nearly 2 years ago all the known blood group systems in man were inherited through genes on the autosomes, that is, on the non-sex chromosomes. Then, at last, a blood group disclosed itself to be carried on the X chromosome (1 2) This was especially lucky because the X is the most interesting of the human chromosomes, in the present state of knowledge.

As you know a normal human being has 23 pairs of chromosomes 22 autosome pairs plus, in the female a pair of Xs and in the male one X and one Y There are about a dozen known blood group systems, and the genes for all of them, with the exception of Xg, are dotted about on the autosomes. We don t know on which autosome any one of them is, but we do know that Xg is on the X.

Mr. And. was patient attending the Butterworth Hospital, Grand Rapids he suffered from nose bleeds due to familial telangiectasia, and the bleeding was so severe that he often had to be transfused. Unfortunately he died from cerebral thrombosis about 6 months ago.

Presumably as result of transfusion Mr And. became immunized, but for some obscure reason he did not make antibody to any well known antigen but made it to previously unrecognized one. The new' antibody anti-Xg^a must be extraordinarily hard to make, for only one other example (3) has since been found though about 20% of transfusions are of the right kind, patient negative, donor positive, to give the patient chance to make anti-Xg^a

The X linkage of the new antigen was noticed first from its behaviour in families and confirmed by its different frequency in the two sexes.

Table I shows our latest count of unrelated white people. This distribution, 65 / of males with the antigen and 89 / of females with it makes sense if two thirds of Caucasian X chromosomes have the gene Xg^a which makes the antigen, and one third have the alternative gene, called Xg which makes no antigen. The allelic gene Xg very likely does make an antigen but one which cannot yet be recognized for lack of the corresponding antibody

Table I

Tests with anti-Xg^a on 1550 unrelated white people, mostly English.

	males	females	gene frequencies
Xg (a+)	65%	89%	Xg^a = 0.661
Xg (a—)	35%	11%	Xg = 0.339

The antigen Xg^a is, like other blood group antigens, a dominant character. One point of finding the gene frequencies is that they can be used to calculate what proportion of children should be Xg (a+) and what proportion should be Xg (a—) in families of the four different mating types. The comparison of the calculated and the observed numbers gives a rigorous check on the theory that the groups are X linked. This is shown in figures 1 and 2. Figure 1 summarizes 178 matings of the types father plus by mother plus. In this and the following figure the plus and minus signs under the Xs represent the gene carried by that X: plus equals Xg^a, the gene responsible for the antigen and minus equals Xg, the silent allele which causes no antigen yet recognized.

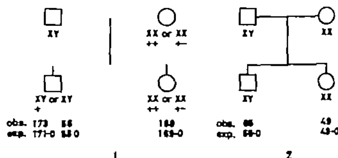


Fig. 1. Issue of 178 matings Xg (+) × Xg (a+).

Fig. 2. Issue of 45 matings Xg (+) × Xg (a—).

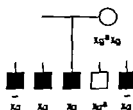


Fig. 3. Family illustrating the linkage between g-6-pd (deficiency shown in black) and the Xg groups (6).

The sons having only one X, and that from their mother can be plus or minus according to whether their mother is homozygous or heterozygous. But all the girls from this mating must be plus because to be a girl they must have their father's X which in this case carries the plus gene, and they all are plus. In truth we have found minus girls from plus × plus matings but one proved to be illegitimate and the others were examples of X chromosome abnormalities. The great excess of boys over girls in this figure is due to selection for families with boys in our linkage studies.

Figure 2 shows the most striking of the matings father plus by mother minus, from it all boys must be minus since their single X comes from their mother and all girls must be plus for they must have their father's X and again they fit. The excess of boys due to deliberate selection shows here too. The other two possible matings also obey the rules.

Having established that the new blood group system was X linked we began to apply it to the mapping of the relative position of genes on the X chromosome. To this end we have been grouping families with other recognizable X-linked characters, such as red green colour blindness, g-6-pd deficiency haemophilia, Christmas disease, Duchenne's type of muscular dystrophy etc. There are many X-linked conditions known in man, and McKusick (4) in a recent publication lists 58 of them.

But here something must be said about the method of measuring the distance between genes. When two genes are sited on different chromosome pairs the two characters they control will appear in the next generation either together or apart depending on chance alone. If on the other hand two genes are on one chromosome pair and are fairly close to each other the two characters they control will be seen travelling together through the generations but occasionally at gametogenesis, at meiosis, crossing-over will happen between the genes and the two characters will part in some of the offspring, such children are called recombinants.

When a pair of genes is on the same chromosome the chance of crossing-over happening between them is greater the further apart they are, so the percentage of recombinant children in a series of families can be used as a measure of the distance between the genes—the higher the percentage of recombinants the further apart the genes. The recombination percentage can be translated directly into what is called map distance by applying a correction.

In normal human beings crossing-over between the two sex chromosomes happens only in the female—between her two Xs there is now thought to be no crossing-over between the X and the Y in the male.

The first Xg linkage to be established was with g-6-pd. Glucose-6-phosphate dehydrogenase deficiency is very rare in northern Europeans but amongst Negroes, certain Mediterranean people and Oriental Jews it is common. Dr Adam in Dr Sheba's department at Tel Hashomer Israel sent samples from 18 families belonging to Iraqi, Kurdish, Yemenite and Sefardic communities, and 11 of them were of the right type to give information and they show that the two genes, Xg and g-6-pd, have a cross-over frequency of about 26 % which corresponds to a distance between them of about 29 map units (5 6, 7) The linkage between Xg and g-6-pd disclosed by these Israeli families has recently been confirmed by a series of Greek families sent to us by Dr George Fraser.

Only certain types of families can give information about crossing-over Figure 3 illustrates the informative type of family the mother is heterozygous for both characters and the destination of all four of her genes is clear the father is left out because he contributes no X to his sons. The mother is heterozygous for g-6-pd for she has given a deficiency allele to 4 of the 5 boys (the 4 marked in black) and a normal allele to one. The mother is Xg (a+) and is proved heterozygous Xg^a Xg by having Xg (a—) sons. Wherever her Xg gene has gone there also has gone her deficiency allele, and her Xg^a gene has gone with her normal g-6-pd allele. In slightly other words the alignment in the Xs of this mother must be Xg gene and g-6-pd deficiency gene on one X and Xg^a gene and g-6-pd normal gene on the other X. There is no recombinant in this family though in most of the families there was at least one. All the other X linked conditions that we have adequately studied show a higher rate of crossing-over with Xg than does g-6-pd.

Now for the problem of the order of genes on the X. The X chromosome is quite a long one, perhaps as long as 175 map units (8) The centromere divides it into a short arm of about 60 map units and a long arm of over 100 units. Figure 4 represents what was known with fair certainty about the relative position of genes on the X before Xg was found. Nothing was known about the position of the many other genes known to be somewhere on the X.

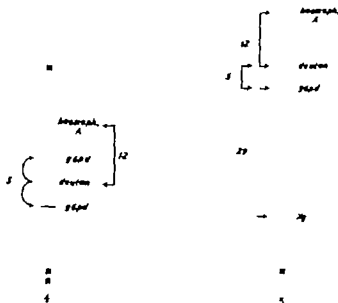


Fig. 4 Linkages between genes on the X known before Xg was found.

Fig. 5. The effect of Xg on the map in fig. 4

This knowledge was within reach because colour vision abnormalities were just frequent enough to appear in some haemophilia A families studied in England by HALDANE AND SMITH (9) and in North Carolina by GRAHAM and his colleagues (10)

Haemophilia A is about 12 map units away from the locus for deutan red green colour blindness. G-6-pd is even closer to deutan, this was found out by ADAM (11) in Israel and by SINISCALCO and his collaborators in Sardinia (12) the probable distance between them was shown, at Johns Hopkins to be about 5 map units (13)

It was not known whether g-6-pd was 5 units from deutan in the direction of haemophilia or away from it because not enough families with both haemophilia and g-6-pd deficiency have been found to allow a direct measurement of recombination between them.

However the linkage of Xg with g-6-pd serves to sort out the probable order of the three genes shown in this diagram in figure 5 Xg is seen to be about 29 map units from g-6-pd. Deutan colour vision is about 5 map units from g-6-pd and it is probably not 5 units towards Xg but 5 units away from Xg for we have consistently found too high a recombination rate between Xg and deutan to be compatible with deutan being nearer to Xg than is g-6-pd. So deutan goes above g-6-pd rather than below

Haemophilia A is about 12 map units from deutan but in which direction was not known. But it must be up too for we are consistently finding too high a recombination rate between Xg and haemophilia for haemophilia to be down below g-6-pd.

The sum of all the available linkage scores (14 15 16 17 and later results) up to the end of July confirm that deutan and haemophilia are further away from Xg than is g-6-pd, and they are confirming that deutan is nearer to Xg than is haemophilia. However the calculated map distances between Xg and deutan and between Xg and haemophilia are so long that we are beginning to suspect that the mathematical translation of human recombination rates into map distance is not yet sufficiently polished to be able to cope with distances over about 30 map units.

We have done the Xg groups of many families with various X borne conditions without detecting any further linkage. And this is perhaps hardly surprising considering the length of the X. A lot of families are needed to detect linkage of two genes when there is 35 % recombination between them and when it approaches 50% linkage can no longer be directly detected. It can be detected indirectly by mutual linkage to an intervening gene.

The negative evidence gathered up to the end of July may be summarized thus The genes for the following conditions are all more than 20 map units away from Xg Duchenne's muscular dystrophy haemophilia, deutan colour blindness, Christmas disease, hypogammaglobulinaemia, hypophosphataemia.

The calculations for Duchenne's muscular dystrophy include families of CLARK et al. (18) as well as those we have tested. It's a pity about Duchenne—a close linkage with Xg would have been extremely useful in the detection of carriers of this dreadful gene.

It can also be said that the genes for the following conditions are not close to Xg renal diabetes insipidus, ectodermal dysplasia, keratosis follicularis, pyridoxine responsive anaemia, deaf mutism,

spastic paraplegia, Hurler syndrome (gargoylism) angiokeratoma, total colour-blindness, chorio-retinal dystrophy

Xg tests on the families of girls with part of one of their two Xs missing made it seem likely that the locus for Xg was sited on the short arm of the X (19) The evidence depended on these abnormal chromosomes being genetically active however current work (20 21) is making it seem unlikely that these abnormal chromosomes are genetically active so we can only say with confidence that Xg is somewhere on the X, we cannot say on which part of the X.

As you know there is a very interesting range of aberrations of the sex chromosomes. In some of these conditions the distribution of the Xg groups is upset. For example, sufferers from the syndrome named after Klinefelter are non-fertile males, who have two Xs and one Y (often they have more than two Xs) Superficially one might expect men with two or more Xs to have an Xg distribution more like that of females. But this is not born out by the 51 we have tested so far and the Xg distribution is somewhere between that of males and females and this is providing food for thought about the origin of these abnormalities.

Then there is Turner's syndrome. Sufferers pass for females but have various things wrong with their gonads and with other parts of their body The basic trouble usually is that they have only one sex chromosome, a single X the sex chromosomes complement of these people is described as XO

That Turner's have only one X could have been discovered from the Xg groups had these been known a few years sooner We have grouped 74 cases of Turner's and their incidence of Xg^a positives, as expected, differs very significantly from the normal female distribution and is very close to the distribution expected in males, and this would have revealed the one X-ness of Turner's had it not already been well known.

We have tested two families with Xg (a+) Turner daughters in which the father is Xg (a+) and the mother Xg (a—) These show that the sole X possessed by a Turner can be her father's X since she can only have inherited her Xg (a+) from him. This represents the first convincing proof (22) that the single X of a Turner's can be paternal in origin belief was mounting that the missing sex chromosome was always the father's fault. A possible explanation is that at one of the divisions at oogenesis the two Xs

that should have parted and gone one to each daughter cell have stuck together and both have gone into the daughter cell destined to be a polar body leaving the cell destined to be the ovum without any X at all. This ovum has been fertilized by an Y bearing sperm. An alternative explanation is that a maternal X has been lost at some early post zygotic cell division.

In conclusion, Xg has made a good contribution, if not as dramatic as we hoped in the first few days of its X linkagehood when, in our ignorance of the length of the X, we supposed that relatively few families would have to be tested to make a quite detailed map of the chromosome. So far Xg has been found to be within measurable distance of g-6-pd, and so of colour vision and haemophilia A, and it has disclosed the order of these genes on the X.

It is hard work mapping a human chromosome the rather thin positive results I have given you represent tremendous activity on the part of our colleagues in collecting about 7 thousand samples of blood. More linkages will doubtless follow but we are beginning to wonder whether *Xg* is near the end of the chromosome it would be bad luck indeed if *Xg* far and away the best marker on the X, were right at one end and wasting half its surveying powers on thin air

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Present Status of Leukemia in Japan with Special Reference to Epidemiology and Studies on the Effect of Chemotherapy

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This paper deals with the present status of leukemia in Japan during the recent 11 years from 1951 to 1961 as studied from the epidemiological point of view as well as the statistical investigation on the effect of new chemotherapeutic agents in the treatment of leukemia. In addition to this, the mode of action of these anti-leukemic agents as observed from their influence upon the nucleic acid synthesis of bone marrow cells and leukemic leukocytes will be reported.

I Incidence of leukemia in Japan

According to the vital statistics of Japan, the annual death rate from leukemia per 100,000 population for all Japan remained at a relatively low level during the 30 year period from 1910 to 1940 being 0.51 in 1910 and 1.3 in 1940 From 1947 it showed a steady increase, being 1.5 in 1950 and 2.8 in 1960 The increase in the incidence of leukemia during the last 10 year period from 1951 to 1960 was more marked than that during the 10 year period from 1941 to 1950 (9 10) The total number of deaths from leukemia was 257 in 1910 939 in 1940 1226 in 1950 and 2628 in 1960. The increase in the death rate from leukemia per 10,000 deaths from all causes was more remarkable, being 2.4 in 1910, 7.8 in 1940, 13.5 in 1950 and 37.2 in 1960. However the annual death rate from leukemia per 100,000 population in Japan is still lower than that in most of other countries. The death rate from leukemia in males was 1.26 to 1.56 times as high as that in females. In the age distri-

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bution curves of the death rate from leukemia in the years 1951 1955 and 1960 there were observed three peaks, namely in the age groups 0–4, 35–49 and 55–69. The increase in the death rate from leukemia was seen in almost all age groups, but the greatest rise was observed in the age group 55–69

In order to know the type incidence of leukemia in Japan, hospital records on cases of leukemia observed at university hospitals and other leading hospitals in Japan during the 6 year period from 1956 to 1961 were collected (11 13) The total number of leukemia cases collected was 3454 These cases were classified as follows according to their cellular type and chronicity acute myeloid 56.8 % acute lymphatic 8.7 % acute undifferentiated 5 9 % (in total acute leukemia 71 4 %) chronic myeloid 19.2 %, chronic lymphatic 2.6 % chronic basophilic and eosinophilic 0 1 % (in total chronic leukemia 21.9 %) and monocytic 6.7 %. Comparing our results with those recently reported in western countries (2 3) two striking differences were found. In Japan, the incidence of acute leukemia is relatively higher than that in western countries, and the incidence of chronic lymphatic leukemia is lower than that in western countries.

II Influence of chemotherapy on survival in leukemia

On 1949 cases of leukemia treated at university hospitals and other leading hospitals in Japan during the last 6 year period from 1956 to 1961 the survival time was estimated from the onset of the first symptom, and the effect of chemotherapy on survival was compared between several groups classified according to cellular types, chronicity and types of treatment. The median survival of acute leukemia classified according to the types of treatment was as follows symptomatic treatment (202 cases) less than 1 0 month, steroid hormone without 6-mercaptopurine (517 cases) 2.9 months, 6-mercaptopurine without steroid hormone (70 cases) 4.2 months, and steroid hormone with 6-mercaptopurine (688 cases) 6.4 months. The median survival of chronic myeloid leukemia classified according to the types of treatment was as follows symptomatic treatment (20 cases) 2.4 years, steroid hormone without busulfan (24 cases) 2.4 years, busulfan without steroid hormone (11 cases) 6.2 years, busulfan with steroid hormone (17 cases) 3.6 years. From these results it was confirmed that the survival time in acute leukemia could be prolonged by chemotherapy with 6-mercaptopurine

or steroid hormone as compared with symptomatic treatment alone, and the combined chemotherapy with 6-mercaptopurine and steroid hormone was more effective than single chemotherapy with 6-mercaptopurine or steroid hormone alone. It was also found that the survival time of chronic myeloid leukemia in patients treated with busulfan was longer than that in those treated with symptomatic treatment alone.

III Influence of antileukemic and antineoplastic agents upon nucleic acid synthesis of bone marrow cells and leukemic leukocytes

A number of antileukemic and antineoplastic agents have been tried for the treatment of leukemia because of their inhibitory effect on cellular proliferation. However only a few studies (1 14) have been reported on the quantitative aspects of nucleic acid metabolism in leukemic cells, and the modes of action of these agents are not yet completely elucidated. In the present study the influence of antileukemic and antineoplastic agents upon nucleic acid synthesis of bone marrow cells and leukemic leukocytes has been observed in vitro by the method described elsewhere (5 6, 7 8, 12, 15 16)

1 The incorporation of formate-^{14}C into nucleic acid bases particularly into DNA thymine by various human leukemic leukocytes and the effect of mitomycin C 6-mercaptopurine and prednisolone Formate-^{14}C was found to be incorporated into the RNA and DNA purines (adenine and guanine) of normal and leukemic leukocytes in vitro (fig 1) The incorporation of formate-^{14}C into DNA thymine was scarcely detected in normal leukocytes and in chronic lymphatic leukemia cells, while DNA thymine was considerably highly labeled in acute myeloid leukemia, acute lymphatic leukemia and chronic myeloid leukemia cells. The DNA thymine of normal and chronic lymphatic leukemia cells was less labeled than the RNA purines, while the DNA thymine of acute myeloid leukemia, acute lymphatic leukemia and chronic myeloid leukemia cells appeared to be much more highly labeled than the RNA purines. There was a statistically significant correlation between the formate ^{14}C incorporation into DNA thymine and the degree of morphologic immaturity of the cell population.

In the case of chronic myeloid leukemia cells mitomycin C, which was originated in Japan and found to be effective in the treatment of chronic myeloid leukemia (4) appeared to suppress

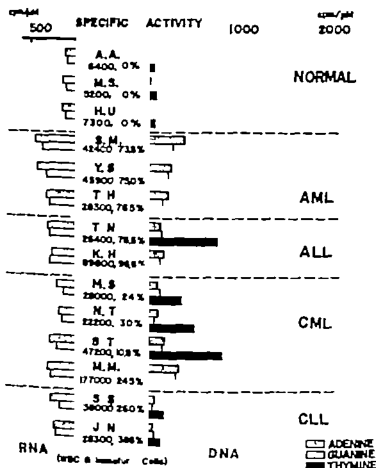


Fig. 1. Incorporation of formate-^{14}C into nucleic acid bases of normal and leukemic human leukocytes in vitro.

the formate ^{14}C incorporation into DNA thymine and DNA purines at the concentrations of 10 and 40 μg/ml, while that into RNA purines was inhibited to a lesser extent at these concentrations. On the other hand, in the case of acute myeloid leukemia cells, mitomycin C (10 and 40 μg/ml) appeared to suppress markedly the formate-^{14}C incorporation into DNA thymine and DNA purines, and that into RNA purines also appeared to be markedly suppressed.

In the case of acute myeloid leukemia cells, 6-mercaptopurine (100 and 300 μg/ml) appeared to affect little the formate ^{14}C incorporation into DNA thymine, while that into RNA adenine and

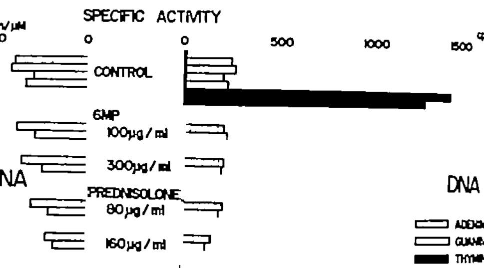


Fig. 2. Effects of 6-mercaptopurine and prednisolone on incorporation of formate-^{14}C into DNA and RNA bases of acute myeloid leukemia leukocytes.

guanine was little or slightly suppressed (fig. 2). On the other hand, prednisolone (prednisolone hemisuccinate, 80 and 160 μg/ml) appeared to suppress the formate-^{14}C incorporation into DNA thymine and that into DNA and RNA purines.

2. *Effect of antileukemic and antineoplastic agents upon the incorporation of formate-^{14}C into nucleic acids of bone marrow cells.* Furthermore, in order to elucidate the effect of various antileukemic and antineoplastic agents upon the nucleic acid synthesis, particularly upon the DNA synthesis of bone marrow cells, the incorporation of formate-^{14}C into nucleic acid bases of rabbit bone marrow cells under the influence of these agents was observed in vitro (fig. 3).

Aminopterin (4 μg/ml) and T-431 L (5-phenylazopyrimidine) (200 μg/ml) suppressed markedly the formate-^{14}C incorporation into DNA thymine, and showed a tendency to suppress that into RNA and DNA purines. It was found that 8-azaguanine (120 μg/ml) did not suppress the formate-^{14}C incorporation into RNA and DNA purines and that into DNA thymine, while 6-mercaptopurine (600 μg/ml) showed a tendency to suppress that into RNA and DNA purines. Both the agents appeared to have no effect upon the incorporation of formate-^{14}C into DNA thymine. Nitrogen mustard N-oxide (methyl [bis-β-chloroethyl] amine N-oxide hydrochloride) (200 μg/ml) and RC-4 (p-phenylenediphosphoric acid tetraethylenemid) (400 μg/ml) suppressed markedly the formate-^{14}C incor-

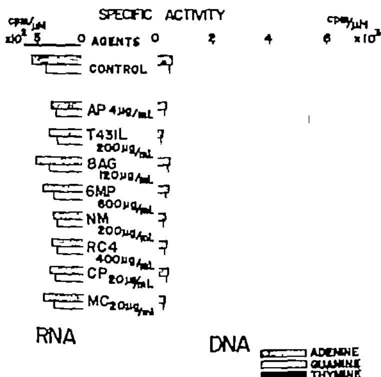


Fig 5 Effects of various agents on incorporation of formate-^{14}C into nucleic acid bases of rabbit bone marrow cells.

poration into DNA thymine, and showed a tendency to suppress that into RNA and DNA purines. Carzinophilin (20 U/ml) suppressed the formate-^{14}C incorporation into DNA thymine and into RNA purine to a similar degree, while mitomycin C (20 μg/ml) suppressed that into DNA thymine and into DNA purine, and showed a tendency to suppress that into RNA purine to a lesser extent as compared with that into DNA thymine.

3. Effect of antileukemic and antineoplastic agents upon the incorporation of adenine-^{14}C into nucleic acids of bone marrow cells and leukemic leukocytes

In an attempt to study further the effect of various antileukemic and antineoplastic agents upon the nucleic acid synthesis of bone marrow cells and leukemic leukocytes, the influence of these agents upon the incorporation of adenine-^{14}C into nucleic acids of rabbit bone marrow cells and leukemic leukocytes was observed in vitro. In normal rabbit bone marrow cells adenine-^{14}C was found to be incorporated mainly into RNA adenine and to a lesser extent into

RNA guanine and DNA adenine. 6-mercaptopurine (100 μg/ml) and 8-azaguanine (150 μg/ml) did not suppress the incorporation of adenine-^{14}C into RNA and DNA. The specific activity of RNA was increased in a concentration of 3 μg/ml of aminopterin. Even in a concentration of 30 μg/ml of aminopterin the incorporation of adenine-^{14}C into RNA and DNA was not suppressed. Nitrogen mustard N-oxide (0.1 and 1.0 mg/ml) suppressed markedly the incorporation of adenine-^{14}C both into RNA and DNA. Endoxan (2 mg/ml) had no inhibitory effect upon the incorporation of adenine-^{14}C into RNA, while it suppressed slightly the incorporation of adenine-^{14}C into DNA. Mitomycin C (40 and 170 μg/ml) suppressed markedly the incorporation of adenine-^{14}C both into RNA and DNA, while prednisolone (60 μg/ml) had no inhibitory effect upon the incorporation of adenine-^{14}C into RNA and DNA.

Vinblastin (0.2 μg/ml) and carzinophilin (100 and 500 U/ml) suppressed markedly the incorporation of adenine-^{14}C both into RNA and DNA. It is interesting to note that chromomycin A_3, a new antineoplastic agent produced by streptomyces griseus No. 7 suppressed selectively the incorporation of adenine-^{14}C into RNA, while it showed no inhibitory effect upon the incorporation of adenine-^{14}C into DNA in rabbit bone marrow cells. Chromomycin A_3 (10 and 50 μg/ml) suppressed markedly the incorporation of formate-^{14}C into RNA purines, while that into DNA purines and DNA thymine was not affected or only slightly inhibited by chromomycin A_3. It also inhibited the incorporation of orotate-^{14}C into RNA uracil and RNA cytosine, while it had no inhibitory effect upon the incorporation of orotate-^{14}C into DNA cytosine and DNA thymine.

The inhibitory effect of antileukemic and antineoplastic agents upon the incorporation of adenine-^{14}C into nucleic acids of leukocytes was also studied in vitro using normal or leukemic leukocytes obtained from the peripheral blood of normal individuals or leukemic patients, respectively. In normal controls, adenine-^{14}C was mainly incorporated into RNA, while the incorporation into DNA was scarcely detected. In chronic myeloid leukemia (1 case) and acute myeloid leukemia (2 cases) the incorporation of adenine-^{14}C into RNA of leukemic leukocytes showed no difference from that in normal controls, while that into DNA was increased as compared with that in normal controls (fig. 4). In chronic lymphatic leukemia (2 cases) the incorporation of adenine-^{14}C into RNA was within

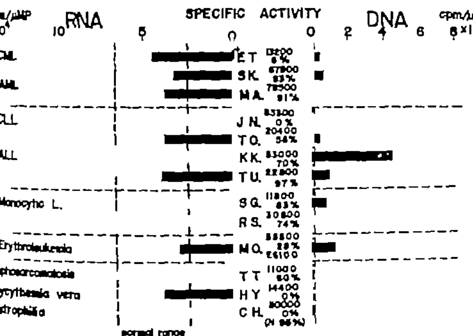


Fig. 4 Incorporation of adenine-^{14}C into nucleic acids of leukemic human leukocytes.

the normal range, and that into DNA was scarcely detected or slightly increased. In acute lymphatic leukemia (2 cases) the incorporation of adenine ^{14}C into RNA was within the normal range or slightly increased, while that into DNA was slightly or markedly increased as compared with that in normal controls. In monocytic leukemia (2 cases) the incorporation of adenine-^{14}C into RNA was within the normal range or moderately increased, while that into DNA was slightly or markedly increased as compared with that in normal controls. There was no close relationship between the immaturity of leukemic cells and the incorporation of adenine-^{14}C into RNA, while the incorporation of adenine-^{14}C into DNA showed a tendency to correlate with the immaturity of leukemic cells.

In a case of acute myeloid leukemia nitrogen mustard N-oxide (1.0 mg/ml) suppressed markedly the incorporation of adenine ^{14}C into RNA and DNA (fig 5). Aminopterin (3.0 μg/ml) suppressed slightly the incorporation of adenine-^{14}C into RNA and inhibited markedly that into DNA. 6-mercaptopurine (100 μg/ml) and mitomycin C (40 μg/ml) suppressed slightly the incorporation of adenine-^{14}C into RNA and DNA. Chromomycin A_3 (10 μg/ml) sup-

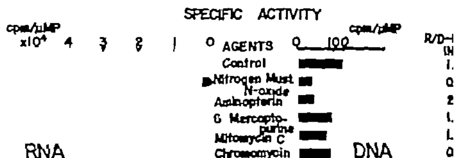


Fig. 5. Effects of antileukemic agents on incorporation of adenine-^{14}C into nucleic ac of acute myeloid leukemia leukocytes.

pressed moderately the incorporation of adenine-^{14}C into RN and inhibited only slightly that into DNA. In a case of acu lymphatic leukemia nitrogen mustard N-oxide (0.1 mg/ml, 1.0 m ml) suppressed markedly the incorporation of adenine-^{14}C in RNA and DNA, especially that into DNA. Aminopterin (1.0 a 3.0 mg/ml) and 6-mercaptopurine (200 μg/ml) inhibited slight the incorporation of adenine-^{14}C into DNA, but they had no i hibitory effect upon the incorporation of adenine-^{14}C into RN Chromomycin A_3 (50 μg/ml) suppressed markedly the incorpor tion of adenine-^{14}C into RNA, and only slightly that into DNA.

In a case of monocytic leukemia, nitrogen mustard N-oxi (1.0 mg/ml) and mitomycin C (40 μg/ml) suppressed marked the incorporation of adenine-^{14}C both into RNA and into DN Aminopterin (3.0 μg/ml) had no inhibitory effect upon the inco poration of adenine-^{14}C into RNA, while it showed a marked inh bitory effect upon the incorporation of adenine-^{14}C into DN 6-mercaptopurine (100 and 250 μg/ml) appeared to have no inh bitory effect upon the incorporation of adenine-^{14}C into DNA an RNA. In a case of chronic myeloid leukemia nitrogen mustar N-oxide (0.1 and 1.0 mg/ml) and mitomycin C (40 μg/ml) su pressed markedly the incorporation of adenine-^{14}C into RNA, whil that into DNA was markedly suppressed by nitrogen mustar N-oxide and slightly inhibited by mitomycin C. 6-mercaptopurin (200 μg/ml) had no inhibitory effect upon the incorporation o adenine-^{14}C into RNA and DNA.

The effect of antileukemic agents upon the incorporation o adenine-^{14}C into nucleic acids of leukemic leukocytes was als studied in course of time in patients with various types of leukemia

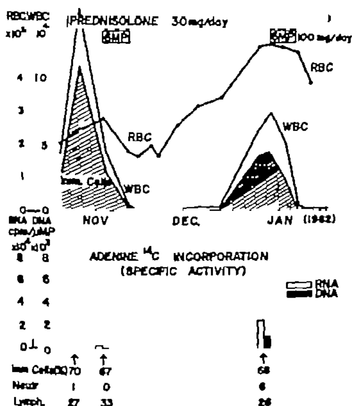


Fig. 6. Clinical course and adenine-^{14}C incorporation into nucleic acids of acute lymphatic leukemia leukocytes.

In a case of acute lymphatic leukemia treated with prednisolone and 6-mercaptopurine, the incorporation of adenine-^{14}C into RNA and DNA was markedly decreased following successful treatment with prednisolone, and it showed a tendency to increase again in parallel with the increase of leukemic leukocytes in the peripheral blood (fig 6) In a case of leukosarcomatosis treated with prednisolone the incorporation of adenine-^{14}C into RNA of leukemic cells was suppressed while that into DNA was not suppressed even when the number of immature cells was decreased in the peripheral blood. In a case of acute myeloid leukemia treated with prednisolone and 6-mercaptopurine, the incorporation of adenine-^{14}C into RNA and DNA of leukemic leukocytes was found to be increased in relapse with the increase of immature cells. It is interesting to note that as shown in the above-mentioned experiments, there is a close

relationship between the incorporation of adenine-^{14}C into nu acids of leukemic leukocytes and the clinical course of the dis in patients with leukemia.

The in vitro studies on the inhibitory effect of antileuk and antineoplastic agents upon the nucleic acid synthesis of b marrow cells and leukemic leukocytes may provide some clue only for the elucidation of the modes of action of these agents also for the selection of suitable chemotherapeutic agents in treatment of leukemia.

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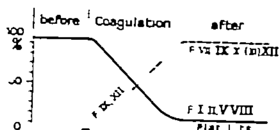
From the Department of Medicine (Head Prof. Dr K. Fellinger)
University of Vienna

Destructive Effect of Lymphnode Cells from Patients with Rheumatoid Arthritis in Tissue Culture

By H Braunsteiner, F Dienstl and M. Eibl

We were able to show recently that 17 of 22 patients with rheumatoid arthritis showed typical skin lesions of the delayed type after having received a homogenate derived from human synovia (2) The histologic picture of the lesion showed perivascular infiltration of mononuclear cells. It can be assumed that delayed hypersensitivity plays an important role in some experimental diseases in rats, guinea pigs and rabbits produced by administration of homologous or heterologous tissue combined with Freund's adjuvant. These diseases, especially encephalomyelitis, thyroiditis and nephritis (4 5 7) could be transfered to recipients by lymphnode cells if the recipient had been made immunologically tolerant to the lymphocytes of the donor (8) It was recently shown that when lymph node cells of animals ill with experimental encephalomyelitis are added to brain tissue culture the glial cells are destroyed

The above mentioned experimental immunologic diseases parallel the so called autoaggressive diseases of humans, like Hashimoto struma, in the microscopic appearance as well as the clinical course of the illness. For this reason we have tried to confirm the above mentioned skin test results by in vitro experiments. First we cultured synovial cells of patients with rheumatoid arthritis and controls to observe the effect of rheumatoid serum and controlserum, as well as of lymph node cells, in the system Unfortunately we found that the growth of synovial cells was too irregular to achieve reliable comparable results. For this reason we attempted to use cultures of human amnion cells as the test material After one to three hours incubation with lymph node cells derived from patients with rheumatoid arthritis, cells from the culture were surrounded and destroyed by these cells.



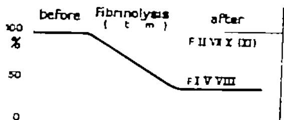


Fig 1

tors to very low levels so that some authors are speaking of a defibrination syndrome ? Only two mechanisms are able to do this *deficient synthesis* of these factors in the liver or *intravascular clotting*. The first possibility can be ruled out with certainty because the defibrination proceeds too rapidly Intravascular clotting is therefore the only plausible explanation in these conditions. At first sight this appears very surprising The assumption that a bleeding disorder might be the consequence of an excessive tendency to clot, in other words of a hypercoagulability was accepted only with reluctance Until recently thrombosing tendency and haemorrhagic diathesis have been considered as being incompatible. But if we take into account that widespread intravascular clotting consumes a significant part of the clotting factors in plasma the bleeding syndrome becomes understandable

Another problem is the relationship between intravascular clotting and fibrinolysis. In fact *fibrinolysis and blood coagulation* represent *two antagonistic systems* blood clotting producing fibrin-olysis destroying fibrin fig ? But although their respective functions are entirely antagonistic the mechanism of both is very similar In both systems a proteolytic enzyme is finally generated thrombin

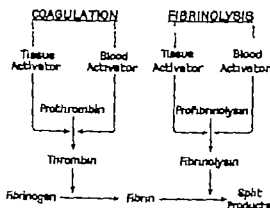
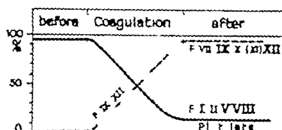


Fig 2.

and fibrinolysin (= plasmin) These enzymes exist in plasma as an inactive precursor prothrombin and profibrinolysin (plasminogen) The activation of this precursor can be achieved in both systems in two ways by a tissue or a blood activator The potency of the tissue activator varies greatly according to the organ many organs produce both a tissue activator of the coagulation and of the fibrinolytic system. The intrusion of tissue juice into the circulating blood may therefore start the clotting as well as the fibrinolytic process. The prostate e. g contains both a clotting activator (tissue thromboplastin) as well as a potent fibrinolytic activator The same is true for the lungs etc. The placenta on the other hand is a very potent source of clotting activator (thromboplastin) but contains no fibrinolytic activator This is of particular importance because the defibrination syndrome was observed first in obstetrics, in premature separation of the placenta, in placenta praevia etc. where the passage of placental fragments with their thromboplastic material into the maternal circulation has been demonstrated by SCHNEIDER et al. That in this way intravascular clotting is produced appears logical, but how can the simultaneous occurrence of enhanced fibrinolysis, which is found regularly in these cases, be explained?

There are apparently *other connections between the two antagonistic systems.* ALEXANDER et al. have demonstrated that thrombin is able to activate profibrinolysin to fibrinolysin in the same way as the tissue and blood activator so that fibrinolysis would have to be considered as a consequence of intravascular clotting Thus seems

INTRA VASCULAR COAGULATION



FIBRINOLYSIS

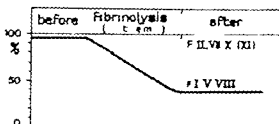


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The above mentioned experimental immunologic diseases parallel the so called autoaggressive diseases of humans, like Hashimoto struma, in the microscopic appearance as well as the clinical course of the illness. For this reason we have tried to confirm the above mentioned skin test results by in vitro experiments. First we cultured synovial cells of patients with rheumatoid arthritis and controls to observe the effect of rheumatoid serum and control-serum, as well as of lymph node cells, in the system. Unfortunately we found that the growth of synovial cells was too irregular to achieve reliable comparable results. For this reason we attempted to use cultures of human amnion cells as the test material. After one to three hours incubation with lymph node cells derived from patients with rheumatoid arthritis, cells from the culture were surrounded and destroyed by these cells.

Methods

Preparation of primary human amnion cultures. Human amnion separated from chorion is washed and pre-digested for 1 hour with 1% trypsin at 37 °C in order to clean it. It is then transferred into 300 ml of 1% trypsin solution and digested for 4 hours. Calf serum is added to the cell-trypsin suspension and after filtration through gauze to remove larger particles it is centrifuged for 10 minutes at 1000 rpm. The supernatant is discarded. The cells are resuspended in medium 199 with 20% calf serum and adjusted to 100,000 cells/ml. Cells are then distributed in test tubes or bottles and cover slips are placed into the suspension to allow histological examination.

Suspension of lymph node cells. Inguinal lymph nodes of patients with rheumatoid arthritis who were not on corticoid therapy are obtained by excision and normal lymph nodes from patients in the course of abdominal operations. The lymph nodes are placed in sterile petri-dishes with a few ml of Hanks solution with 20% calf serum and subsequently centrifuged for 10 minutes at 1000 rpm. The supernatant is discarded, the cells resuspended with the same solution, filtered through gauze to get rid of large particles and washed again and counted. 1—3 x 10^6 lymph node cells/ml are added to 4-6 day old cultures of human amnion. Before adding the lymph node cells the medium is discarded after having shaken the bottles gently to dislodge cells not sticking firmly to the surface. To some of the bottles fresh guinea-pig serum is added to study the effect of complement on the system. One, three and 24 hours after incubation cover slips are removed and prepared with the May-Grunwald stain. At the same time lymph node cells in the supernatant are counted.

Results

1—3 hours after addition of lymph node cells of patients with rheumatoid arthritis to the cultures, adherence of these cells to amnion cells could be observed. Aggregates of 5—10 cells appeared at a later stage (fig. 2). The peak of this reaction occured in about



Fig. 1. Culture of amnion cells.



2 3

Fig 2. Aggregates of lymphnode cells around amnion cells.

Fig 3. Destruction of amnion cells by lymphnode cells.

three hours. Later the number of aggregates decreased and after 24 hours no aggregates were detected. At this time, only a few lymphnode cells were left in the supernatant of the culture fluid. Aggregation occured more often around single cells and it could not be detected in the complete cell sheet. Only a small percentage of amnion cells of a culture, probably from the sites where the cell sheet is not complete, is involved in the composition of aggregates. After aggregation has reached a certain point, possibly after a certain time, amnion cells are destroyed. First vacuolation and granulation of the cytoplasm occurs, then lysis of the nucleus takes place (fig. 3).

No aggregation can be seen if lymphnode cells of controls are added to the cultures (fig. 1).

Discussion

From these results, it is evident that various amnion cells are surrounded and destroyed in cell cultures by lymphnode cells taken from 5 patients with rheumatoid arthritis, whereas lymphnode cells of normal persons show no such effect. From the morphological

point of view the active cells correspond to small and medium-sized lymphocytes.

At early time periods our observations correspond to the investigations of KOPROWSKI AND FERNANDES (6) who added lymphnode cells of rats with experimental encephalomyelitis to cell cultures of glia-cells, as well as to that of ROSEMAN AND MOON (10) who added lymphocytes of sensitized animals to the cells used as antigen. With late observation, however destruction of cells is much less conspicuous in our work than in the two above mentioned investigations, so that after 24 hours amnion cell cultures seem to be completely intact again and lymphnode cells have disappeared. It seems as if, by improved culture methods of synovia cells, more specific results could be achieved. PULVERTAFT et al (9) have already reported a special affinity of lymphocytes in vitro to human thyroid gland cells in cases of Hashimoto-struma. BROBERGER AND PERLMANN (3) by tests with isotopes, showed a destruction of intestinal cells in cell cultures in cases where lymphocytes of patients with colitis ulcerosa were added. By personal information a destructive effect of lymphnode cells of patients with PCP on synovia cells was reported, but no detailed data were given (11)

No definite information is available on the mechanism of the affinity of lymphnode cells for certain organs apparently considered as antigens. According to ROSEMAN AND MOON (10) serum factors are irrelevant the effect being most apparent in synthetic medium. According to investigations of KOPROWSKI AND FERNANDES (6) however lymphnode cells of normal animals can also destroy glia cells if the incubation takes place in the presence of serum of animals with experimental encephalomyelitis. It is obvious to draw a parallel between lymphnode cells in cell cultures and mononuclear cells in the mononuclear infiltrate present in the cases of experimental encephalomyelitis as well as in cases of rheumatoid arthritis. The histologic picture shows a definite vacuole around the invading mononuclear cells in cases of Hashimoto-struma. In cases of encephalomyelitis, myelin is destroyed up to a circle of 20 μ surrounding the round cells (11) Similar observation have been made in the mononuclear infiltrate of transplanted pieces of skin. We have recently discussed the functional relations of sensitized lymphocytes and perivascular round cells (1) It is probable that sensitized lymphocytes either appear directly in the mononuclear infiltrate or pass on immunologic information to mononuclear cells.

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The Mechanism of the Hemostatic Function of Blood Platelets

By A. HELLEM AND P. A. OWREN

By in vivo microscopy of transparent living tissue in animals the following sequence of reactions can be visualized taking part in the formation of hemostatic plugs

1 Within 1–3 seconds after injury of a small vessel, the platelets start to adhere to the damaged endothelial cells at the edge of the vessel wound and to tissue fibres particularly to collagen, which are exposed on the wound surface (3 10)

2 New platelets adhere to those already fixed and to each other forming loose platelet aggregates which ultimately cover the opening of the vessel These aggregates are permeable to the outflowing blood which passes through channels. These may be clogged, but new channels are steadily formed.

3 After some minutes all channels become definitely clogged and the bleeding stops. In the light microscope, the granular platelet clumps seem then to have largely fused into a more or less structureless mass with disappearance of platelet outlines. This process was called viscous metamorphosis by EBERTH AND SCHIMMELBUSCH in 1886 (4) By electron microscopy has been found that structural changes occur in the platelets with loss of granula and mitochondria. The membrane however is well preserved of most of the platelets (8)

4 The last step is visible fibrin formation by coagulation of plasma which is observed a little later The fibrin reinforces and consolidates the hemostatic plug but seems to be of secondary importance only and not essential for provisional primary hemostasis. The three first steps, in which platelets have a key position, will be discussed in more detail

1 Platelet adhesion to the wound surface The mechanism of adhesion of platelets to collagen fibres is unknown The process is independent of calcium since it is not prevented by strong solutions of citrate or by EDTA. The reaction can be conveniently studied therefore by suspension of washed collagen particles in EDTA plasma followed by microscopy of the sediment. The aggregation reaction is then blocked. We have found that the adhesion to collagen takes place normally in plasma from patients with von Willebrand s disease and with thrombasthenia, and it is not blocked by mono iodo acetic acid. The essential properties of the surfaces which attract platelets are not well understood.

2. *The reversible aggregation reaction* In 1958, HELLEM (7) discovered a dialysable and heat stable factor in red blood cells which in minute amounts produces platelet aggregation and changes the normal non adhesive platelets into adhesive platelets. This substance has later been identified as adenosine diphosphate by GAARDER et al. (6) The reaction is blocked by calcium binding agents. Aggregates produced by ADP are reversed by removal of calcium. The required calcium concentration is low since aggregation may be produced in plasma with the usual citrate concentration. It decreases however with increasing citrate concentrations and it is abolished by EDTA.

The mechanism of aggregation has not been clarified. A possible explanation suggested by LALAND (11) of the role of calcium as a binding link between the platelets would be that ADP if at

Fig 1 Suggested mechanism for platelet aggregation by ADP Willebrand factor and calcium.

tached to the platelet surface will have three negative valencies for the binding of calcium (fig 1) Two of these would be occupied by one calcium ion the third would bind only one valence of a calcium ion and the other would be available for the remaining free valence of ADP attached to the surface of an other platelet. This mechanism would explain the lack of aggregation by adenosine monophosphate and adenosine triphosphate, as demonstrated by BORN (2) because AMP has two free valencies and ATP has four for the binding of calcium and consequently there will be no available free valence for the calcium bridge If this theory is correct, adenosine tetraphosphate which have five free valencies, should act like ADP We have recently found that this substance aggregate platelets in the same manner as ADP provided the increased calcium requirement is considered. This finding strongly supports the calcium bridge theory

The aggregation reaction requires viable platelets with normal glycolysis. This concept is based on the following observations By repeated washings or by prolonged storage, the platelets become non-reactive to the addition of ADP Aggregation is also prevented by iodo-acetate and iodine which block glycolysis. The addition of fluorid, however increases the reactivity to ADP Since iodo-acetate and fluorid act at different stages in the glycolysis at aldolase and enolase respectively it is reasonable to believe that reactions between these two stages are of importance for maintaining a correct surface of the platelets for the attachment of ADP

That ADP is attached to the surface of the platelets seems likely since the reaction is inhibited by addition of AMP and ATP as shown by BORN (2) We have found that the reaction is blocked also by adenine and adenosine The NH_2 group therefore, seems to take part in the attachment reaction The inhibition by these substances seems competitive, since renewed addition of ADP in larger amounts will reverse the reaction The ADP platelet reaction is also competitively inhibited by addition of cysteine ethylmercaptan, cysteamine and other substances containing free SH groups. The OH group in position 2 of the ribose part of ADP also seems to be involved because deoxy-adenosine-diphosphate does produce aggregation only in high concentration. All the substances mentioned above which inhibit the aggregation reaction also greatly prolong the bleeding time if the wound is flushed by solutions of these substances.

Thrombasthenia is the only clinical syndrome in which aggregation is lacking by addition of ADP in excess of 0 2 μ/ml to citrated platelet rich plasma. This observation may be used as a simple diagnostic test as illustrated in fig 2 In the normal plasma the aggregation is very marked 30 seconds after the addition of ADP whereas in the thrombasthenia plasma no aggregation is seen.



Fig. 2 The addition of ADP produces marked aggregation in normal plasma (N) but no aggregation in thrombasthenia (P)

ADP for the progressive aggregation reaction during hemostasis can probably not be provided by the damaged tissue cells. HOVIG (9) demonstrated that adenosine diphosphate is released from platelets by contact with collagen fibres. Since platelets are rich in ADP it is reasonable to believe therefore that platelets are self-supporting ADP for progressive aggregation ZUCKER AND BORRELLI (17) demonstrated a platelet aggregating effect also of saline extracts of tendon and subcutaneous fat This effect may probably be explained by residual particles of collagen in the extracts used.

In von Willebrand s disease the bleeding time is prolonged and the platelet consumption at the wound surface is defective (1) probably because of defective platelet aggregation. In vitro, the ad

dition of larger amounts of ADP to platelet rich plasma from such patients produces grossly normal aggregation, but on the addition of small amounts an abnormal reaction occurs as first demonstrated by Vainer and Caen (16). We have confirmed this finding. The addition of ADP to a final concentration of 0.05 μ/ml plasma produced no increased platelet adhesiveness in plasma from von Willebrand's disease in contrast to an increase to about 25 % adhesive platelets in normal plasma and in plasma from patients with different coagulopathies (table I).

Table I

The addition of ADP to a final concentration of 0.05 μg/ml produces no aggregation in von Willebrand plasma in contrast to a marked aggregation in normal plasma and in plasma with coagulation disturbances.

	Adhesive Platelets		
A.D.P. γ/ml	0.10	0.05	0.025
Normal (average)	51	25	12
Von Willebrand's disease (5)	40	—	—
Hemophilia A (3)	53	35	17
Hemophilia B (3)	51	29	13
Anticoagulant treated pat. (3) TT 5 Hematuria	57	30	17

The abnormal response of platelets to ADP in these patients is corrected in vitro by addition of citrated platelet poor plasma from normal individuals, particularly after muscular exercise and by plasma from diabetic patients. These findings strongly support the concept of Nilsson et al. (12, 13) that von Willebrand's patients are lacking a plasma factor which is necessary for normal hemostasis. Egeberg (5) in our laboratory demonstrated that muscular exercise in von Willebrand's patients not only results in an increase in factor VIII activity but also in a decrease of the bleeding time. Transfusion of normal blood to such patients has only a moderate effect on the bleeding time, whereas transfusion of blood from diabetic patients or from normals collected after vigorous exercise for some minutes may completely restore the bleeding time to normal (fig. 3). This observation is of value for the treatment of haemorrhagic episodes in such patients. Patients may also be operated without abnormal bleedings after such transfusion.

The platelet aggregation which is produced by addition of ADP to normal plasma reverses spontaneously. This is probably due to destruction of ADP by enzymes in plasma. The optimal

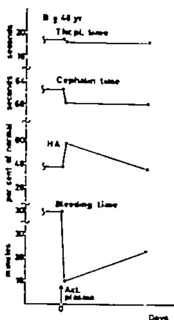


Fig. 3. Transfusion of exercise acti'ated plasma from normal donor shortens the bleeding time in patient with von Willebrand' disease to normal.

temperature for the inactivation reaction is about 37 C. The effect is destroyed by heating of the plasma to 58 C for 15 minutes.

The third phase in the hemostatic reaction is irreversible platelet aggregation and viscous metamorphosis which produces impermeability of the platelet plug and arrest of bleeding. This reaction requires thrombin which we believe is provided by clotting factors which are adsorbed on to the platelet surface and not by the coagulation of plasma. This is evidenced by the following experiments. Haemophiliacs are characterized by a prolonged secondary bleeding time. Transfusion of a concentrate of normal platelets to hemophilia A restores the secondary bleeding time to normal (14 15). Fig 4 illustrates such an experiment. The secondary bleeding time, which exceeded 30 minutes, came down to normal and reverted only slowly to the pretransfusion level in about 6 days. The plasma level of factor VIII in these patients remained below 1 / during the whole period. The platelet suspension used contained a small amount of plasma, but the transfusion of an identical amount of platelet free plasma from the same donor had no effect.

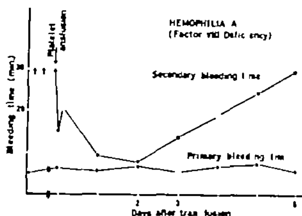


Fig. 4 Transfusion of platelet concentrate to patient with hemophilia A shortened the secondary bleeding time to normal. The effect lasted for several days. The factor VIII concentration in plasma did not change.

The hemostatic effect therefore, must be ascribed to factor VIII being adsorbed on the transfused platelets.

In factor V deficiency the results of transfusion of normal platelets were similar. In hemophilia B the effect of platelet transfusion on the secondary bleeding time lasted only a couple of hours. A similarly shortlasting effect was observed on the *primary* bleeding time in a case of factor X deficiency produced by intensive anticoagulant therapy. These differences in the duration of effect may be explained by the in vitro finding that factors IX and X are only loosely adsorbed and easily eluted from the platelets, whereas factors VIII and V are strongly adsorbed and can not be removed by washing.

All these findings strongly support the concept that the coagulation reactions which are responsible for the hemostatic effect as measured by the primary and the secondary bleeding time test, take part at the surface of the transfused platelets. We therefore believe that the platelet surface clotting systems have a key role in hemostasis and should be regarded as separate clotting systems which from a functional point of view are partly independent of the plasma coagulation systems. This also explains that provisional platelet hemostasis may be normal in spite of incoagulable plasma such as in congenital fibrinogen deficiency and after administration of large doses of heparin.

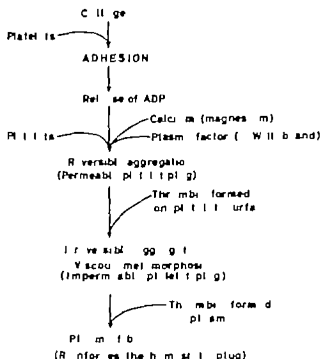


Fig. 5. Theory for the mechanism of hemostasis.

Based on these investigations we are suggesting the schema for the hemostatic mechanism presented in fig 5

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Proc. 9th Congr. europ. Haemat., Lisbon 1963 Acta haemat. 31 239–246 (1964)

Department of Medicine, University of Basle

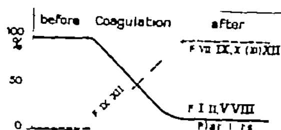
Intravascular Clotting and Spontaneous Fibrinolysis

By F. Koller

The interest in *spontaneous fibrinolysis* has increased considerably in the last few years since we use *therapeutic fibrinolysis* on a larger scale. In most instances enhanced spontaneous fibrinolysis is just an accidental finding without clinical significance occurring in a variety of conditions such as cirrhosis of the liver and other hepatic disorders, neoplastic diseases including leukaemias etc.

However in relatively rare cases spontaneous fibrinolysis is associated with an extremely *severe haemorrhagic diathesis* the patients often bleeding to death in a very short time. What happened in these cases? The degree of fibrinolysis is often not markedly different from that found in patients without manifest bleeding tendency. This observation has thrown some doubt on the importance of fibrinolysis in the pathogenesis of the bleeding syndrome mentioned. Since we employ artificially induced fibrinolysis for the treatment of thrombotic conditions we know that even marked fibrinolysis is usually well tolerated in patients with no recent injury. Another mechanism must therefore be involved in this particular bleeding tendency. It was logical to look for a *simultaneous coagulation or platelet defect* and in fact markedly decreased values of fibrinogen, factor V and factor VIII were repeatedly detected. But these changes could still be produced by fibrinolysis (fig. 1). If the latter is sufficiently activated it does not only dissolve fibrin but also attacks the chemically closely related factors I (fibrinogen) V and VIII. In some cases however also a marked decrease of prothrombin (factor II) and of platelets was demonstrated, a finding which cannot be explained by fibrinolysis alone.

What is then the mechanism which—besides fibrinolysis—reduces the concentration of fibrinogen and some other clotting fac



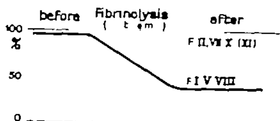


Fig 1

tors to very low levels so that some authors are speaking of a defibrination syndrome? Only two mechanisms are able to do this *deficient synthesis* of these factors in the liver or *intravascular clotting*. The first possibility can be ruled out with certainty because the defibrination proceeds too rapidly. Intravascular clotting is therefore the only plausible explanation in these conditions. At first sight this appears very surprising. The assumption that a bleeding disorder might be the consequence of an excessive tendency to clot, in other words of a hypercoagulability was accepted only with reluctance. Until recently thrombosing tendency and haemorrhagic diathesis have been considered as being incompatible. But if we take into account that widespread intravascular clotting consumes a significant part of the clotting factors in plasma the bleeding syndrome becomes understandable.

Another problem is the relationship between intravascular clotting and fibrinolysis. In fact *fibrinolysis and blood coagulation* represent *two antagonistic systems*: blood clotting producing fibrin, fibrinolysis destroying fibrin (fig 2). But although their respective functions are entirely antagonistic the mechanism of both is very similar. In both systems a proteolytic enzyme is finally generated: thrombin

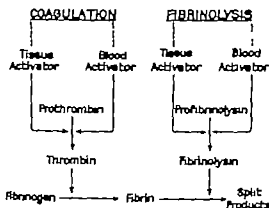


Fig. 2.

and fibrinolysin (=plasmin) These enzymes exist in plasma as an inactive precursor prothrombin and profibrinolysin (plasminogen) The activation of this precursor can be achieved in both systems in two ways by a tissue or a blood activator The potency of the tissue activator varies greatly according to the organ many organs produce both a tissue activator of the coagulation and of the fibrinolytic system. The intrusion of tissue juice into the circulating blood may therefore start the clotting as well as the fibrinolytic process. The prostate e. g. contains both a clotting activator (tissue thromboplastin) as well as a potent fibrinolytic activator The same is true for the lungs etc. The placenta on the other hand is a very potent source of clotting activator (thromboplastin) but contains no fibrinolytic activator This is of particular importance because the defibrination syndrome was observed first in obstetrics, in premature separation of the placenta, in placenta praevia etc. where the passage of placental fragments with their thromboplastic material into the maternal circulation has been demonstrated by SCHNEIDER et al. That in this way intravascular clotting is produced appears logical, but how can the simultaneous occurrence of enhanced fibrinolysis, which is found regularly in these cases, be explained?

There are apparently *other connections between the two antagonistic systems*. ALEXANDER et al. have demonstrated that thrombin is able to activate profibrinolysin to fibrinolysin in the same way as the tissue and blood activator so that fibrinolysis would have to be considered as a consequence of intravascular clotting This seems understandable also from a teleological point of view The organism

tries to counteract intravascular clotting by activating the antagonistic system. If this is logical in theory it is disastrous in fact. During intravascular coagulation certain clotting factors (factors I II, V VIII) as well as the platelets are consumed other factors behave like enzymes, participating in the clotting process without being changed and still others are even activated during clotting (fig 1) If we consider only the factors consumed, we realise that—with the exception of prothrombin—the same factors are also attacked by strongly activated fibrinolysis. Furthermore the split products of fibrinolysis interfere with fibrin polymerisation so that haemostasis is even more impaired. The combination of intravascular clotting and fibrinolysis aggravates therefore the clotting defect and explains that a particularly severe haemorrhagic diathesis is the consequence.

It is usually impossible to decide—on the basis of clotting analysis—what is more important in a given case intravascular clotting or fibrinolysis. The latter is easily recognized, therefore often misdiagnosed as primary fibrinolysis, as the only abnormality and treated in consequence, not to the benefit of the patient! It is therefore an important diagnostic rule that spontaneous fibrinolysis associated with a severe haemorrhagic diathesis is usually combined with intravascular clotting, particularly if the platelet count and the prothrombin concentration are low Intravascular clotting is probably more frequent than was hitherto assumed.

In fact *any thrombosis* represents an intravascular clotting. Freisly has demonstrated that even a localised thrombosis produces regularly a moderate and temporary but distinct fall of the platelet count. These changes are not sufficient to interfere with haemostasis. In order to become of clinical significance, intravascular clotting must be widespread or even generalised. The latter condition manifests itself clinically in 2 entirely different forms

Intravascular clotting may produce *widespread thrombosis involving usually veins and arteries* leading to large necrotic areas, which become haemorrhagic, as a consequence of the depletion of the plasma of certain clotting factors—or it may produce a generalised but *invisible coagulation* no thrombus, no coagula are seen only thin fibrin threads are formed which may at worst occlude very small vessels or are deposited as a fine layer on the vascular endothelium. Nevertheless clotting factor are consumed, the plasma again be-

comes depleted of them, and spontaneous bleeding in the skin and inner organs occurs. The reason for this difference in clinical appearance is not entirely clear. Experimental work going back to WOOLRIDGE in 1886 and others shows that slow intravenous infusion of tissue thromboplastin produces an invisible intravascular clotting, whereas rapid infusion of the same material is followed by the formation of large coagula occluding veins and pulmonary arteries.

I should like to present briefly a clinical example of either form which we observed recently.

1. Purpura fulminans: A 50 years old woman was admitted to the hospital with haemorrhagic gangraena of both legs which in a few days spread to the thighs and to the arms. Fibrinogen was 35 mg% (according to CLAUSS method). Prothrombin varied between 34 and 58%, factor V between 6 and 35%. Factor VII was 100%. Platelets 95,000 (In retrospect it is evident that the factor V deficiency which we described in 1950 in purpura fulminans was due to intravascular clotting). In spite of all our therapeutic efforts the patient expired. At autopsy multiple, almost generalised thromboses of veins and arteries were found, the latter causing the symmetric gangraena of the extremities. Histological examination revealed marked infiltration of the vessel wall, generalised vasculitis probably of allergic origin. Purpura fulminans being observed in the majority of cases (not in ours) as a complication of scarlet fever may be considered as an equivalent to post-scarlatinous nephritis or rheumatic fever which are also of allergic origin.

2. Waterhouse-Friderichsen-Syndrome. A 53 years old woman very suddenly fell ill with fever, multiple skin-haemorrhages, slight stiffness of the neck, collapsus and coma. An acute meningococcal septicaemia could be demonstrated. Fibrinogen 70 mg%, prothrombin 64%, factor V 11% (controlled value). Thrombocytes 68,000. Within a few hours the patient died. No thromboses, no coagula were found at autopsy.

In both patients an intravascular coagulation could thus be demonstrated, with and without production of visible clots. The pathogenesis of intravascular clotting is probably similar to that of localized thrombosis. VIRCHOWS triad seems to be valid too, namely 1. lesion of the vessel wall, 2. slowing of the blood stream, 3. alteration of the blood itself, i. e. hypercoagulability.

ad 1. Lesions of the vessel wall was quite evident in our first patient with purpura fulminans and generalised vasculitis probably of allergic origin.

ad 1 and 2. A more discreet vascular lesion combined with slowing of the blood stream due to shock has to be assumed in our 2nd patient with Waterhouse-Friderichsen-Syndrome. It is probable that this clinical syndrome is related to the experimental Shwartzman-Sanarelli phenomenon in which LASCH has clearly demonstrated an intravascular coagulation. Finally the fibrin deposits in extra

corporeal circulation are probably due to the non physiological artificial vessel wall

ad 3 The most important of the 3 points stressed by VIRCHOW is undoubtedly the alteration of the blood itself, in other words hypercoagulability which may be produced by a variety of conditions by intrusion of placental fragments or of amniotic fluid (which are rich in thromboplastin and in fibrinolytic activator) in the maternal circulation during surgical procedures in the lungs, the prostate, the pancreas etc. where tissue juice rich both in thromboplastic and fibrinolytic material may be poured into the circulating blood the same can occur in neoplasms, especially metastasizing tumors of the prostate, the gallbladder the stomac, the lungs, the pancreas, and in malignant melanoma. In accute haemolysis due to transfusion of incompatible blood or other causes the potent thromboplastic material of the erythrocytes discovered by QUICK and confirmed by LEUPOLD in our laboratory is mixed with plasma, starting intravascular coagulation.

This phenomenon is therefore by no means exceptional, especially if we learn to recognise its early stages which is important

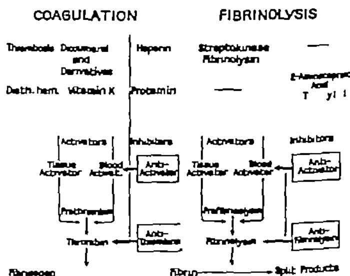


Fig. 2

for the *therapeutic approach*. In order to get a clear idea of our possibilities I have represented in fig 3 the various compartments of the clotting and fibrinolytic system which must be considered. Both systems, coagulation and fibrinolysis, have their inhibitors and here again an astonishing similarity can be demonstrated between the two systems. Today pharmacological agents are available which influence profoundly the various components of the two systems. The activity of certain clot promoting factors is increased by vitamin K, decreased by dicoumarol and its derivates heparin produces a potent inhibitor of the clotting process. The fibrinolytic system is activated at wish by streptokinase, urokinase or fibrinolysin, and finally epsilon-aminocaproic acid and Trasylol act as powerful inhibitors of fibrinolysis.

If a patient is admitted to the hospital with a severe haemorrhagic diathesis and presents by routine examination an enhanced fibrinolysis, there is a temptation to give an inhibitor of fibrinolysis, e. g. epsilon-aminocaproic acid in order to correct this abnormality As already mentioned it is very improbable that the severe bleeding tendency is caused by spontaneous fibrinolysis alone a simultaneous intravascular clotting must be assumed. For this abnormality *heparin* would be the treatment of choice. One is of course reluctant to give heparin to a patient with severe bleeding I think, however that we are allowed and even obliged to do so under the following conditions

The diagnosis of intravascular clotting must be substantiated by determination of the fibrinogen (using a clotting method, not a chemical one) which must be very low and by a platelet count. Before heparin is given the patient receives a fibrinogen preparation, to which epsilon-aminocaproic acid or Trasylol is added in order to avoid an immediate breakdown by fibrinolysis. Heparin is given thereupon by continuous intravenous drip in amounts just sufficient to prolong slightly the whole blood clotting time (or thrombin time) compared with the values before the beginning of heparin infusion. In this way intravascular clotting should be stopped. Fibrinogen regeneration proceeds rather rapidly attaining normal values in about 24 hours.

With this heparin treatment many patients could be saved, which is especially important as the conditions described (with the exception of neoplastic diseases) are all potentially curable.

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J. F. A. P. Miller und P. Dukor: Die Biologie des Thymus nach dem heutigen Stande der Forschung. S. Karger AG Basel/New York 1964. VI + 98 S., 32 Abb., 8 Tab., Preis sFr 20.—

Die Initiative zur Publikation der vorliegenden Monographie ging von Dr. P. Kallós, Hälsingborg, aus, der auch das Vorwort verfasst hat.

Die beiden kompetenten Autoren Miller und Dukor geben in übersichtlicher Darstellung das heutige Wissen über die Thymusfunktion wieder, sie diskutieren bei der Behandlung der vielen noch offenen Fragen alle Hypothesen mit Angabe einer vollständigen Dokumentation und berichten über zahlreiche eigene Forschungsergebnisse. In der Phylogenese und in der Ontogenese fällt der Zeitpunkt der ersten spezifischen Immunreaktionen mit dem Erscheinen der Lymphocyten zusammen. Die allerersten Lymphocyten treten im Thymus in Erscheinung, und das Thymusgewebe stellt die älteste Lymphocytenproduktionsstätte des Körpers dar. Bei der neugeborenen Maus verhindert eine radikale Entfernung des Thymus die normale Entwicklung des lymphatischen Systems. Die beträchtliche Grösse des Thymus in frühen Lebensstadien, die später auftretende Thymusatrophie und die Beobachtung, dass sich epitheliale Thymuszellen in Lymphocyten differenzieren können, lassen es, wie die Autoren vorsichtig formulieren, als möglich erscheinen, dass der Thymus mindestens teilweise den Ursprungsort der lymphatischen Zellen des Organismus darstellt. Die lymphatischen Zellen sind bekanntlich keine einheitliche Population. Das Thymusgewebe unterscheidet sich histologisch von andern lymphatischen Organen durch seine epithelialen Zellen, durch die Hassalschen Körperchen, die etwa siebenmal grössere Mitosehäufigkeit, das Fehlen von Keimzellen und die sehr geringe Zahl von Plasmazellen. Die Ausschwemmung von Thymuslymphocyten und ihre Ansiedelung in andern Organen des lymphatischen Systems sind bisher allerdings noch nicht bewiesen. Es kann aber berechnet werden, dass der Thymus weit mehr Lymphocyten produziert als für sein eigenes Wachstum erforderlich wären. Ein humoraler, im Thymus produzierter Faktor, wie etwa ein die Lymphocytenproduktion oder die Lymphocytenfunktion stimulierender Faktor, konnte weder durch die Arbeiten der Autoren noch durch diejenigen anderer Forscher mit hinreichender Sicherheit nachgewiesen werden, wenn auch gewisse Versuche mit Diffusionskammern für die Existenz eines acellulären Thymusfaktors sprechen. In diesem Zusammenhang wird die Abgabe von Desoxyribonucleinsäure-Bestandteilen als Informationsträger diskutiert.

Ein besonderer Abschnitt behandelt die Reaktionen des Thymus auf physikalische und chemische Einflüsse, auf Hormone, Antigene und pathogene Viren. Von besonderem Interesse sind die Kapitel über experimentelle Befunde beim thymektomierten Tier. Diese weitgehend von den Autoren selbst durchgeführten Arbeiten sind durch sehr schöne histologische Bilder belegt und haben zu einer entscheidenden Bereicherung unserer Kenntnisse über die Thymusfunktion geführt. Die neonatale Thymektomie hat bei der Maus eine Verarmung des ganzen Körpers an kleinen Lymphocyten zur Folge und beeinträchtigt die normale Entwicklung der immunologischen Abwehrmechanismen. Homo- und manchmal auch Heterotransplantate werden nicht abgestossen oder überleben doch wesentlich länger als bei scheinoperierten Kontrolltieren. Andere Experimente zeigen, dass auch die Regeneration des lymphatischen Systems nach Ganzkörperbestrahlung thymusabhängig ist. Beim neonatal thymektomierten Tier können die Ausfallserscheinungen durch Injektion von Lymphocyten, durch Übertragung suspendierter Thymuszellen und durch Thymusimplantation behoben werden.

Ein letzter Abschnitt behandelt die Pathologie primärer Thymuserkrankungen des Menschen, und ein umfangreiches Literaturverzeichnis beschliesst die Monographie, deren Lektüre jedem immunologisch oder allgemein hämatologisch interessierten Leser angelegentlich empfohlen werden kann.

H. R. Marti, Basel

R. K. Archer The Eosinophil Leucocytes. Blackwell, Scientific Publications Ltd., Oxford. 205 p., 10 fig., Price: 40 s.

A reader desiring background of information on eosinophils would find the book decidedly deficient. References to outstanding papers and reviews, such as those of OPIE, SCHWARTZ, [illegible], RUD, CAMPBELL, TATAI AND OKADA, G. T. ARCHER (who is currently working with horse eosinophils) were noticeably lacking. The author stated in the preface that his review of the literature covered work on eosinophils prior to 1955, but even this review was limited. The majority of the book was centered around experiments performed by the author since 1956, mostly to studies with horse eosinophils.

According to ARCHER intradermal injections of histamine into horses produced local edema and an accumulation of eosinophils. Similar local accumulations of eosinophils occurred when the histamine was injected into bone marrow Prior injections of whole eosinophils, suspensions of eosinophil granules, or water extracts of eosinophils reduced the edema and the local eosinophilia. Similar inhibitory effects were obtained if the skin was pretreated with eosinophil extracts prior to injections of 5 hydroxytryptamine or antigen.

These and related observations led ARCHER to speculate that chemotactic reactions to histamine account for most of the known facts about eosinophils. He suggests that the release of eosinophils from the bone marrow is determined by blood histamine levels and that eosinophils migrate out of the blood vessels into tissues containing increased amounts of free histamine. He presumes that eosinophils accumulate in inflammatory areas around mast cells which have discharged their granules or at the site of antigen-antibody reactions where histamine is also released. Since eosinophil extracts contain antihistamine activity it is postulated that they inactivate or detoxify histamine in the tissues.

It is the re iewer opinion that great many difficulties have to be resolved before such broad interpretations can be accepted. Archer indicated that the antihistamine potency of eosinophil extracts varied from pony to pony and in some little or no antihistamine activity was present. Moreover the action of eosinophil granules or eosinophil extracts never produced complete inhibition but merely reduced the intensity of the local reaction if injected immediately prior to relatively small doses of histamine (10 μg) The used eosinophil suspensions were never completely free of other cells such as neutrophils and basophils.

Experiments relating eosinophils to parasitic infestations, as well as local eosinophilia to intradermal injections of antigen in immunized horses were described, along with those dealing with the action of ACTH and cortisone. Methods for the separation of eosinophils from human and horse blood, and procedures for skin biopsy staining of cells, etc., were described. The discussions of hemopoesis, comparative morphology, and pathology were superficial. ROBERT S. SPEIRS, New York

Obituary Notice

It is with great regret that we announce the sudden death on March 30, 1964, of Dr SHIGEYASU AMANO, Kyto University Kyoto (Japan)

Acta haemat. 31: 249–261 (1964)

Department of Medicine, Cambridge University, Cambridge

Plasma Factors in Leukaemia

Immunological and in vitro- Cultural Studies

By D. C. Cowling, D. Quaglino and F. G. J. Hayhoe

Despite a great deal of experimental work and hypothetical speculation there still exists no clear or established concept as to the role of immunological factors in the pathogenesis of leukaemias or their influence in processes of remission and relapse. Surveying the published reports on antigenic and immunological changes in leukaemia Hayhoe (13) concluded that the picture at that time was a thoroughly confused one: leukaemic cells might sometimes have additional or different antigens to those of normal leucocytes, but might also lack important antigens normally present: leukaemic cells might be sufficiently foreign in structure to stimulate antibody formation, or might themselves elaborate globulins with antibody activity against normal body constituents. More recent studies have continued to yield conflicting results. Gorer et al. (9) working with chemically induced and spontaneous leukaemia in mice, were able to protect against transmitted leukaemia by passive immunization with adsorbed sera prepared by immunizing foreign strains of mice with leukaemic tissue. This suggested the presence of foreign antigens (X antigens) in the leukaemic cells. Garb et al. (7) succeeded in preparing specific sera against human leukaemic cells in rabbits, again implying the existence of specifically leukaemic antigens in the cells concerned. Harrington et al. (12) reported satisfactory depression of leucocyte counts and occasional improvement in skin infiltration following immunization of patients suffering from chronic granulocytic leukaemia, using their own cells with Freund's adjuvant after treatment with busulphan. Concurrently with clinical and haematological improvement a fall in the serum complement level was observed. The authors suggested that prior

administration of busulphan caused antigenic alteration of leukaemic cells, so that better immune responses could be obtained, a suggestion which seems to imply that antigenic change results from the action of the alkylating agent rather than arising de novo. Absence of specific antigenic change in leukaemic cells is supported by the work of MOLONEY et al. (19) who used several immunological methods but found no antileukaemic cell antibodies in rats with transmitted myelogenous leukaemia.

With regard to levels of plasma factors having immunological importance, studies in leukaemia again show a lack of uniformity in results. Leucocyte antibodies have sometimes been present in acute leukaemia in man but often not (25 1) complement levels have usually been normal, but sometimes not (24 2 12) properdin levels have sometimes been low especially in lymphocytic leukaemia, but frequently normal (6 23 14 3)

Although the lack of consistent patterns either in antigenic characteristics of leukaemic cells or in immunological components of leukaemic sera is discouraging we considered it possible that some advance might be made by the use of short-term 'in vitro culture methods, which would allow us to compare the dynamic behaviour of normal and leukaemic cells in their own and exchanged plasmas. A series of immunological studies including measurement of serum complement levels, immuno-conglutinin titres and tests for the presence of leuco-agglutinins and complement fixing antibodies against freshly prepared suspensions of normal and leukaemic leucocytes were therefore undertaken in a group of leukaemic patients. The rate of incorporation of ^{3}H thymidine in leukaemic cells cultured over 72 hours in their own plasma or in normal serum was observed to see if any alteration in behaviour over a period of time related to plasma factors could be determined. The behaviour of normal cells in their own and leukaemic plasma was also observed.

Serological Studies

Methods

The serum complement and serum immuno-conglutinin levels were performed by the methods of Coombs et al. (5) Sera were separated and frozen at —20 °C on the day of taking the blood. The tests were usually performed within few days of taking the blood on serum samples thawed only once. Leucocyte suspensions were prepared from group 0 defibrinated blood, either leukaemic or normal, using siliconized glassware and glass beads. Dextran was added to aid sedimentation of red cells, and after initial centrifugation of the leucocyte rich plasma for 7 minutes at 800 r.p.m. and 4 °C

the supernatant was removed and to haemolyse erythrocytes 6 ml of distilled water was added for 20 seconds, the tubes being shaken. After 20 seconds 2 ml of 3.4% saline was added and the tubes again gently shaken. The cells were then washed twice in saline, centrifuging as above, and the final concentration adjusted to 5—10,000 leucocytes per mm³. The suspensions were used on the day of preparation. For the leucoagglutinin test, sera were inactivated, and to serum dilutions of 1:4 and 1:8 the leucocyte suspension was added in equal volumes. In each case normal leucocytes were used, and in many cells from a type of leukaemia like that of the subject providing the serum were also employed (table I). The test was read microscopically following tipping the tubes after incubation at 37 °C for 1 hour. The complement fixation tests were carried out using a dropping pipette. The patients' inactivated sera were used at 1:4 and 1:8 dilutions, and the fresh suspension of leucocytes prepared as above was used as antigen. Two M.H.D. of complement was used. Antigen and serum controls were included with each serum tested, and a complement control for each batch of tests.

Results

Serum complement determinations were carried out on 20 young and middle-aged fit adults. They were not matched for age and sex with the leukaemic patients. The mean titre was 1:170, with a range of 1:98 to 1:238. Serum complement levels were measured in 24 leukaemic patients, tests being repeated in several cases at intervals of 7—21 days during a period of observation. The results are shown in table I.

In five cases of acute leukaemia the mean titre taking the highest reading from any one case was 1:146 with a range of 1:78 to 1:238. In case 1 three consecutive readings at ten day intervals showed an appreciable rise in titre. The patient during this period showed little clinical or haematological change, was treated with 6-mercaptopurine and prednisone, and had a large blood transfusion.

In seven cases of chronic granulocytic leukaemia the mean titre taking the highest reading from any one case was 1:138 with a range of 1:98 to 1:191.

In 12 cases of chronic lymphocytic leukaemia the mean titre, taking the highest reading from any one case, was 1:138 with a range of 1:62.5 to 1:238 (table I). No correlation between the degree of control of the leukaemia and the level of complement was observed.

The immune-conglutinin levels in 20 normal controls were nil in all but one, who had a titre of 1:16. Coombs et al. (5) found normal values fluctuated, but of 6,000 blood donor sera approximately 10% had a titre above 1:4. Of the 24 leukaemic patients one case

Table I
Serological studies in leukaemic patients

Case No.	Treatment	Complement titre	Immuno-conglutinin titre	Leuco-agglutinins Normal cells	Leuco-agglutinins Leukaemic cells	Leucocyte C.F. Test Normal cells	Leucocyte C.F. Test Leukaemic cells	Comments
	Acute leukaemia.							
1	Prednisone 40 mg/day	) 122	—	—	—	—	—	I full relapse
	6 M.P 100 mg/day	b) 153	—					
		c) 238	—					
2	Prednisone 40 mg/day	) 122	—	—	—	—	—	Had multiple boils. In full relapse
	6 M.P 100 mg/day	b) 153	4					
3	Prednisone 40 mg/day	78	—	—	—	—	—	Almost complete remission Pustules on legs
4	Prednisone 40 mg/day	98	—	±1:16	—	—	—	Partial remission
	6 M.P 100 mg/day							
5	Prednisone 40 mg/day	153	—	—	—	—	—	Complete remission
	Chronic granulocytic leukaemia.							
6	Busulphan 2 mg/day	153	—	—	—	—	—	Well controlled
7	Busulphan 2 mg/day	98	—	—	—	—	—	Well controlled
8	DXR to spleen	153	—	—	—	—	—	Partial remission
9	Busulphan 4 mg/day	98	—	±1:8	—	—	—	Partial remission
10	Off busulphan	153	—	—	—	—	—	Well controlled
11	Prednisone 20 mg/day	) 153	—	—	—	—	—	Undergoing blastic transformation
	6 M.P 100 mg/day	b) 191	—					
12	Off busulphan	122	—	—	0	—	0	Well controlled

	Chronic lymphocytic leukaemia							
13	Chlorambucil 2 mg/day	a) 98	—	—	0	—	0	Count rising and glands enlarging
		b) 191	—					
14	Off chlorambucil	a) 238	—	—	—	—	—	Leukaemia controlled
		b) 153	—					Urinary tract infection
15	Prednisone 20 mg/day	) 178	—	—	0	—	0	Leukaemia deteriorating
		b) 98	—					Massive splenomegaly
		) 62.5	—					Cachexia
		d) 191	—					
16	Chlorambucil 2 mg/day	98	—	—	0	—	0	High count. Poorly controlled. Thrombocytopenia
17	Chlorambucil 4 mg/day	122	—	±1:8	—	—	—	Well controlled. No past transfusion
18	Chlorambucil 3 mg/day	98	—	—	0	—	0	Well controlled
19	Prednisone 5 mg/day Chlorambucil 2 mg/day	78	—	—	—	—	—	Well controlled
20	Chlorambucil 4 mg/day	78	—	—	0	—	0	Well controlled
21	Chlorambucil 5 mg/day	98	2	—	—	—	—	Well controlled
22	Chlorambucil 5 mg/day	153	—	—	0	—	0	Well controlled
23	Prednisone 15 mg/day Chlorambucil 1 mg/day	122	—	—	—	—	—	Well controlled
24	Prednisone 20 mg/day	) 153 b) 191	—	—	±1:8	—	—	Well controlled

— = negative result. 0 = test not done with leukaemic cells.

of chronic lymphocytic leukaemia had a titre of 1 2 and from a case of acute leukaemia one of two serum samples had a titre of 1 4 This latter patient suffered from staphylococcal sepsis at this time.

Leuco-agglutinins were present in four patients, one with chronic granulocytic leukaemia, one with acute leukaemia, and two with chronic lymphocytic leukaemia. These patients had not been transfused.

Complement fixation tests were negative in all instances.

Tissue Culture Studies

Methods

Short-term tissue cultures of leucocytes from normal and leukaemic subjects were set up as previously described (22) but phytohaemagglutinin (PHA) was invariably omitted since we were concerned with possible slight stimulatory or inhibitory effects of different plasmas, which might ha e been obscured by the powerful stimulatory action of PHA on some cells. Final cell counts were adjusted to between 1,500 and 2,000 leucocytes per mm^3 The leukaemic cell cultures were set up using the patient's plasma and normal compatible plasma or usually group AB serum. In one case of acute myeloblastic leukaemia and one case of chronic lymphocytic leukaemia normal leucocytes from a subject of the same ABO group were set up in the leukaemic plasma and their own plasma.

Cellular activity in DNA synthesis was studied by removing one ml aliquots of the cultures at 0, 24 48, and 72 hours, and incubating them for 1 hour at 37 °C with H-thymidine, used at a final dilution of 0.5 μc/ml. The aliquot was then centrifuged and smears were made from the deposit and fixed with methanol for subsequent staining. Autoradiographs were prepared using Kodak AR10 stripping film which was left in contact for six days prior to development. The smears were then counterstained by May-Grunewald Giemsa stain. Differential counts were made on 100 or sometimes 200 cells, and those with thymidine uptake scored as follows. 5—10 grains = 1; 11—20 grains = 2 21—35 grains = 3 36 and more grains = 4. The final score was obtained by adding the individual ratings of 100 consecutive cells, the possible range being 0—400.

Results

Normal cultures Two normal leucocyte cultures were performed, the cells being set up both in their own plasma and in leukaemic plasma. In table II are set out (a) the percentage of nucleolated cells in which are included lymphoid blast cells and prolymphocytes (b) the percentage of cells showing ^{3}H thymidine uptake (c) the ^{3}H-thymidine score. Virtually no blastic transformation or thymidine uptake took place in any of the experiments with normal leucocytes.

Acute leukaemia. Three cases of acute myeloblastic leukaemia and one of acute lymphoblastic leukaemia were studied. The results

Table II

Culture	Day examined	Leukaemic plasma 0	1	2	3	Normal plasma 0	1	2	3
Normal cells	% of blast cells (plasma from acute myeloblastic case)	0	0	3	3	0	0	1	1
	% showing ³HT uptake	0	0	0	0	0	0	0	0
	³HT score	0	0	0	0	0	0	0	0
Normal cells	% of blast cells (plasma from chronic lymphocytic case)	0	3	1	3	0	2	1	3
	% showing ³HT uptake	0	0	0	0	0	0	0	0
	³HT score	0	0	0	0	0	0	0	0

are set out in table III where are shown (a) the percentage of primitive cells up to and including the prolymphocyte and myelocyte stages, (b) the percentage of all cells showing ^{3}H thymidine uptake and (c) the ^{3}H thymidine score. The results show that the proportion of primitive cells whether initially high or low did not increase on culture, but in one instance (case 26) showed a slight fall. Thymidine uptake was present from the commencement of culture and tended to increase progressively in the two cases of acute myeloblastic leukaemia with high initial blast cell counts. In the third case where the initial blast cell count was low there was no such progressive increase, and in the case of acute lymphoblastic leukaemia a sharp fall was manifest by 48 hours. While the overall pattern of behaviour in all these cultures was generally similar whether the cells were incubated in their own or in normal plasma, slight differences in case 25 were noted which might indicate a diminished thymidine uptake when the patient's plasma was exchanged with normal plasma.

Blastic crisis of chronic granulocytic leukaemia two cases were examined and the results of case 28 are tabulated in table IV In this case the substitution of the patient's plasma by normal serum did cause enhanced thymidine uptake, an effect not shown in the second case.

Chronic granulocytic leukaemia. Two cases were examined and the results of case 7 are tabulated in table IV Case 30 with a low count of immature cells showed no increase in thymidine uptake over the period of culture and no enhancement of uptake in normal serum, but case 7 with a higher count of immature cells, showed some

Table III

Acute leukaemia.

Patient	Day of Examination	Patient' Plasma 0	1	2	3	Normal Serum 0	1	2	3
11	% myeloblasts and myelocytes	94	95	90	92.5	94	95.5	92.5	96
Acute myeloblastic	% showing ^{3}H thymidine uptake	1	7.5	16	28.5	3	1.5	15.5	32.5
	^{3}H thymidine score	3	28	63	111	12	6	59	125.5
25	% myeloblasts and myelocytes	77	80	72	81	85	72	85	88
Acute myeloblastic	% showing H thymidine uptake	7	12	24	23	8	18	11	17
	^{3}H thymidine score	27	48	96	92	30	72	42	67
26	% myeloblasts and myelocytes	14	13	3	5	11	8	9	6
Acute myeloblastic	% showing ^{3}H thymidine uptake	6	4	2	3	3	3	5	3
	^{3}H thymidine score	22	14	7	10	11	10	20	11
27	% lymphoblasts	100	100	99	100	100	100	100	100
Acute lymphoblastic	% showing ^{3}H thymidine uptake	6	6	1	0	7	9	0	0
	H thymidine score	18	21	3	0	24	25	0	0

Table IV

Patient	Day examined	Leukaemic Plasma 0	1	2	3	Normal Serum 0	1	2	3
28	% of blast cells	93	98	99	99	98	100	100	90
Acute on chronic	% showing ^{3}HT uptake	7	12	8	17	10	34	25	19
granulocytic leukaemia	HT score	25	43	25	67	38	135	97	94
7	% of myelocytes and myeloblasts	71	66	32	24	71	68	59	33
Chronic granulocytic	% showing ^{3}HT uptake	20	32	11	4	18	27	21	10
leukaemia	^{3}HT score	77	127	44	15	71	100	80	39
31	% of blast cells	0	0	0	0	0	0	0	0
Chronic lymphocytic	% showing ^{3}HT uptake	2	1	1	0	1	1	0	0
leukaemia	^{3}HT score	2	3	1	0	2	1	0	0
34	% of blast cells	0	0	1.5	3	0	1	1	2
Chronic lymphocytic	% showing ^{3}HT uptake	0	0	0	0	0	0	0	0.5
leukaemia	HT score	0	0	0	0	0	0	0	[illegible]

increase in uptake at 24 hours in both cultures and a slower rate of fall-off in synthetic ability at 48 to 72 hours in the culture with normal serum. In case 7 it was also noted that the proportion of blast cells fell during the time of culture and this was associated with an increase in the mature forms.

Chronic lymphocytic leukaemia. Two cases were examined case 31 untreated with a high lymphocytosis, and case 34 treated and in partial remission with a nearly normal leucocyte count. The results are tabulated in table IV No thymidine uptake of significance took place. The substitution of normal plasma for the patient's plasma did not influence thymidine uptake.

Mitotic activity in all these cultures was very scant Virtually no mitotic figures were observed in the acute leukaemias until 48 to 72 hours, and none in the chronic leukaemias.

Discussion

Our findings confirm those of previous workers regarding the level of serum complement in leukaemia namely that it may be normal or slightly lowered. The patients studied had all received chemotherapy There seemed no correlation between the degree of remission induced and the serum complement level Nevertheless, in view of the observations of HARRINGTON et al. (12) previously quoted it seemed logical to look for complement fixing antibody against normal and leukaemic leucocytes in treated cases. As leuco-agglutinins have been reported in leukaemia (15) and may be associated with complement fixing antibody to leucocytes (18) leuco-agglutinin tests were undertaken. No complement fixing antibodies to a suspension of freshly prepared intact leucocytes were detected although occasional cases had positive leuco-agglutinin tests.

COOMBS et al (5) have suggested that immuno-conglutinin may be regarded as an auto-antibody capable of reacting with complement fixed within the host, and its production may be stimulated by complement fixed in vivo Raised levels of immuno-conglutinin have been demonstrated in such conditions as rheumatoid arthritis and acute hepatitis (5) and multiple sclerosis and Hashimoto's disease (4) If complement played a role in any immune reaction responsible for remission in leukaemia one might

expect to find raised immuno-conglutinin titres, but we have not found this.

The uptake of isotope labelled substrates by leukaemic cells has been studied by several workers. LAJTHA et al. (16) cultured marrow aspirate from 3 to 24 hours with ^{32}P orthophosphate. He showed that blast cells from acute leukaemia have a strikingly lower rate of DNA synthesis than promyelocytes and myelocytes from normal marrow, and no difference was found whether normal or the leukaemic plasma was used in the cultural medium. GAVOSTO et al. (8) also found that far fewer leukaemic myeloblasts were labelled when incubated for 1, 3 or 5 hours with ^{3}H thymidine than were myeloblasts from normal marrow. HALE AND COOPER (11) also using ^{3}H thymidine, incubated with leucocytes from peripheral blood for half an hour, observed that some acute leukaemic cells show a delay in DNA synthesis. We have found no reports of the sequential capacity for thymidine uptake of cells maintained in culture for three days nor any accurate assessment of the mitotic activity under these conditions although following the addition of colchicine adequate numbers of metaphase spreads are often obtained.

The studies here reported have indicated that leukaemic cells from peripheral blood cultures show scant mitotic activity, usually less than 1%, as compared with the greater mitotic activity usually seen in normal lymphocytes stimulated with phytohaemagglutinin. However, during the period of culture immature cells of the myeloid series particularly showed a progressive increase in the numbers showing capacity for ^{3}H thymidine incorporation. Mature cells, that is those not capable of division, whether in leukaemic or normal blood, showed no thymidine uptake.

The finding in one patient with acute myeloid leukaemia, that when cells were incubated for two or three days in their own plasma they may then have a greater capacity for ^{3}H thymidine incorporation than when incubated in normal serum suggests that this technique may be a sensitive one in detecting plasma factors affecting cellular activity. Whether this particular effect is due to a growth stimulating substance in leukaemic plasma of the type recorded by METCALF (17) or whether it would indicate a growth controlling substance in normal serum, we cannot differentiate in these preliminary studies.

Similarly the opposite finding in one case of acute blastic crisis of chronic granulocytic leukaemia of improved thymidine uptake over the period of culture when the cells were incubated in normal serum may relate to a plasma factor. It could be that such factors may affect cells of different types in different ways. However in this patient (case 28) we cannot exclude the effect of therapeutic agents in her own plasma as at this time she was receiving 6-mercaptopurine and prednisone.

We considered it worth while incubating normal cells with leukaemic plasma as various substances, phytohaemagglutinin (20), bacterial antigens (21) and leucocyte anti-sera (10) have been shown to cause dedifferentiation of lymphocytes and thus allow ^{3}H thymidine incorporation. No such effect was observed with the two leukaemic plasmas used.

While no evidence of immune body activity in states of leukaemic remission has emerged from this study, the techniques used may provide a relatively sensitive means of detecting plasma factors and other environmental influences which may operate to stimulate or inhibit proliferative activity in normal or pathological cells. The diverse cytological and clinical pictures among the leukaemias might lead one to expect a lack of uniformity in the results of experiments relating to cell growth and reduplication in various media, but the search for consistent patterns of cellular or humoral activity in specific varieties of leukaemia is likely to be facilitated by extension and refinement of the cultural methods here described.

Summary

No complement fixing antibody against normal or leukaemic leucocytes was detected by serological tests in 24 patients suffering from leukaemia. The serum complement levels in leukaemic patients were not markedly lower than in normal control subjects. No increased incidence of immuno-conglutinin was detected.

In acute myeloblastic leukaemia, blast cells in culture often showed an enhanced capacity for ^{3}H-thymidine incorporation following incubation for 24 to 72 hours in culture, but mitotic activity was scant. When the capacity of cells to incorporate ^{3}H-thymidine after culture in their own plasma or normal serum for 24—72 hours was compared, minor differences were observed. In one case of acute myeloblastic leukaemia enhanced uptake occurred following incubation in the patient's plasma and in one case of blastic crisis of chronic granulocytic leukaemia the reverse was noted, with enhanced uptake in normal serum. In both acute lymphoblastic leukaemia and chronic lymphocytic leukaemia in remission or relapse, thymidine uptake was small and fell sharply over the period of culture.

Zusammenfassung

Bei 24 Patienten mit Leukämie konnten mit serologischen Methoden keine komplementfixierenden Antikörper gegen normale oder leukämische Leukozyten nachgewiesen werden. Im Vergleich zu normalen Kontrollpersonen war der Komplementgehalt des Serums bei Leukämiepatienten nicht deutlich vermindert. Ebenso war die Häufigkeit von Immun-Konglutinin nicht größer.

Bei akuter Myeloblastenleukämie zeigten die Blasten in der Kultur oft ein gesteigertes Vermögen zum Einbau von H^3 Thymidin nach einer Inkubationsdauer von 24 bis 72 Stunden. Ihre mitotische Aktivität war jedoch gering. Beim Vergleich der Einbaurate von H^3 Thymidin nach Kultur im eigenen Plasma oder in Normalserum während 24 bis 72 Stunden ergaben sich geringfügige Unterschiede. Bei einem Fall von akuter Myeloblastenleukämie war die Aufnahme nach Inkubation im Patientenplasma gesteigert, und bei einem Fall von myeloblastischer Krise einer chronischen myeloischen Leukämie war das Gegenteil festzustellen, nämlich eine gesteigerte Aufnahme im Normalserum. Sowohl bei akuter Lymphoblastenleukämie als bei chronischer lymphatischer Leukämie in Remission oder im Rückfall war die Thymidinaufnahme gering und fiel während der Dauer der Kultur scharf ab.

Résumé

Chez 24 malades atteints d'une leucémie la présence d'anticorps fixateur du complément contre les leucocytes normales ou leucémiques n'a pas pu être démontrée à l'aide de méthodes sérologiques. En comparaison avec des personnes normales, le titre de complément du sérum des malades atteints de leucémie n'est pas diminué notablement. De même la fréquence de l'immuno-conglutinine n'est pas plus grande.

L'incorporation de la thymidine H_3 des blastes d'une culture cellulaire des leucémies myéloblastiques après une incubation de 24 à 72 heures est souvent plus active, alors que leur activité mitotique est faible. La comparaison de l'activité de l'incorporation de la thymidine H_3 après la culture dans leur propre plasma ou dans un plasma normal ne révèle que de faibles différences. L'incorporation après incubation dans le sérum du malade était plus active dans un cas de leucémie aigue à myéloblastes, alors que le contraire était observé dans un cas d'une crise à blastes d'une leucémie myéloïque chronique, c'est-à-dire une plus forte activité dans le sérum normal. Lors des rémissions ou des rechutes des leucémies aigues à lymphoblastes et des leucémies lymphatiques chroniques, l'incorporation de la thymidine était faible et décroissait sensiblement pendant la durée de la culture.

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Über die Fibrinfibrillenbildung und Fibrinquerstreifung

Von A. Fonio Chur

Die Definition des Fibrins, des Endproduktes des Gerinnungsvorganges hat in den letzten Jahrzehnten sowohl durch die lichtoptische als durch die elektronenoptische Forschung einige Wandlungen durchgemacht. Die lichtoptische und die Betrachtung im Dunkelfeld des Fibrinausfalles im Deckglaspräparat in Nadel-Faden- und Netzform wurden im letzten Jahrzehnt durch die Dunkelfeldmikroskopie am freien Fibringerinnsel ergänzt, wodurch der Werdegang des Fibrinausfalles in seine verschiedenen Phasen bis zur Retraktion und das weitere Verhalten des Fibrinendproduktes bis zur fibrinolytischen Auflösung ermöglicht wurden.

Die Dunkelfeldbetrachtung läßt die verschiedenen Phasen vom Ausscheiden des Fibrins bis zur Retraktion unterscheiden und im Bilde darstellen das Ausscheiden der kompakten Fibrinmasse, die zu Beginn zwar eine Andeutung von Gerinnungsstreifen aufweist, nachfolgend jedoch völlig strukturlos erscheint und den Proteinaufbau der Fibrinfäden und der Fibrinfibrillen, die bündelförmig zusammengerückt die Fibrinstränge bilden. Beide finden sich auf der kompakten strukturlosen Fibrinmasse freiliegend, teilweise oder gänzlich davon überdeckt Die Fibrinstränge durchziehen die Fibrinmassen auf weite Strecken hin, ähnlich den Eisenstangen eines «armierten Betons» einer Baustelle.

Die Fibrinquerstreifung als Vorläufer und Endausgang der Retraktion findet sich nur in den Fibrillen und deren Strängen vor während die kompakte, strukturlose Fibrinmasse keine Retraktionsvorgänge aufweist, sie wird von den sich retrahierenden Fibrillen und Fibrillensträngen passiv mitgerissen so zum retrahierten Fibringerinnsel in toto führend.

Nach der Beschreibung der Untersuchungsmethoden werden die Phasen bei Gerinnung und Retraktion in zwei Abschnitten behandelt über den Proteinaufbau und die Querstreifung der Fibrinfibrillen.

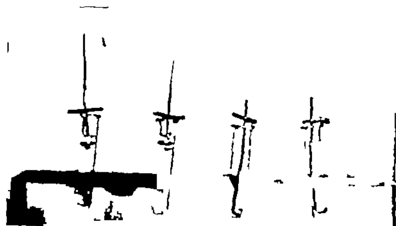


Abb. 1 Gerät zur Bestimmung der Retraktion im Blutplasma. 4 Mikroretraktionsröhrchen (2,5 cm lang, 0,5 breit), je 2 Silberstempelchen mit waagrecht torquiertem und Hackenende (zur Unterkühlungsmethode)

Methoden

Um die verschiedenen Phasen des Fibrinausfalles bzw. der Fibrillenbildung und der nachfolgenden Retraktion zu erfassen, die im Citratplasma des normalen Blutes zu rasch erfolgen, als daß sie zur mikroskopischen Untersuchung erfaßt werden können, muß das Citratplasma gerinnungsgehemmt werden.

Der Befund von Querstreifung in den Fibrinmaschen des langsam gerinnenden Plasmas im Liquor cerebrospinalis bei tuberkulöser Meningitis von WOLPERS und RUSKA erstmals und von uns später in den Fibrillen von langsam gerinnendem hämophilen Plasma erhoben, bewog uns normales Plasma zu »hämophilisieren«. Durch Zusatz von winzigen Mengen von Heparin zum Citratplasma gelingt es seine Gerinnung und Retraktion derart zu verlangsamen, daß es gelingt, die gewollten Phasen zur Untersuchung zu erfassen. Der gleiche Effekt kann durch Unterkühlung des Blutes und seines Plasmas ohne Zusatz erreicht werden.

Heparinisierungsmethode

1. Blutentnahme: 9 ml Blut zu 1,0 Natr. citric. 2,5 %, mischen.

2. Zentrifugieren 3 Min. lang bei 2500 Min. Touren.

3. Abheben der Plasmaschicht.

4. Erstellen der Heparinlösung: 1 ml 40° Liquemin-Lösung zu 50 mg Heparin in 100 ml physiologische Kochsalzlösung. 1 ml davon = 0,5 mg Heparin und in 0,01 ml = 0,005 mg Heparin.

5. Citratplasma 0,5 ml + 0,01 ml Heparinlösung mit 1 Tropfen $CaCl_2$ 2% rekalzifizieren.

6. Gerinnen und retrahieren lassen in Retraktionsröhrchen bei Zimmertemperatur.

Präparierung der Retraktionsröhrchen: 1. mechanische Reinigung mit warmem Wasser und Seife, 2. mit Vel-Lösung behandeln, 3. mit heißem Wasser nachspülen, 4. mit Aq. dest. nachspülen, 5. bei Wärme trocknen lassen, 6. untere Hälfte der vorbereiteten Gläschen mit Bunsenbrenner ausglühen. Nur bei derart vorbereiteten Gläschen erfolgt die Retraktion ungestört ohne Ankleben an der Glaswand.

7. Herausnehmen des Gerinnsels und Teilverbringung unter Deckglas zur Dunkelfeldbetrachtung.

Unterkühlungsmethode

1. Vorgängiges Abkühlen der Zentrifugierröhrchen in Eisgefäß und im Kühlschrank bei — 5° C.
2. Blutentnahme von 10 ml Blut durch korrekte Venenpunktion.
3. Unterkühlen der mit Blut beschickten, abgekühlten Röhrchen 20 Min. lang bei — 5° C.
4. Zentrifugieren 5 Min. lang bei 2500 Min. Touren.
5. Abheben der Plasmaschicht und Verbringung mittels feiner Kanüle in abgekühlte Mikroretraktionsröhrchen. Silberstempelchen mit rundem, horizontal torquiertem Ende an den oberen Teil der Plasmasäule verbringen und eines weiteren Silberstempelchens mit Hackenende an den Grund des Mikroröhrchens (Abb. 1).

Gerinnen und retrahieren lassen bei Zimmertemperatur.

6. Nach erfolgter Gerinnung oder bei beginnender Retraktion je nach der gewollten Phase

a) am horizontal torquiertem Stempelchen anklebendes Gerinnsel in toto unter Deckglas bringen.

b) über dem Hackenstempelchen lagerndes Gerinnsel mit feiner Pinzette über das obere Ende gleiten lassen und unter Deckglas verbringen.

7. Direkte Betrachtung im Dunkelfeld ohne Präparierungsmaßnahmen.

Über den Proteinaufbau der Fibrinfibrillen

Bei der lichtoptischen Betrachtung des Fibrinausfalles im Deckglaspräparat nimmt man wahr, wie zunächst einzelne Fibrinnadeln oder Fibrinfäden ausfallen und nach und nach an einem zerfallenden Thrombozyt oder Granulahäufchen konzentrisch oder direkt an einem solchen entstehen und bei weiterem Fortschreiten des Fibrinausfalles zum Fibrinnetz sich einordnen, das ganze Blickfeld einnehmend. Bei anderen Präparaten fallen lange Fibrinfäden aus zu einem weiten Fibrinnetz angeordnet, ohne konzentrische Einstellung nach Thrombozyten. Bei Betrachtung im Dunkelfeldmikroskop des Deckglaspräparates verhält sich der Fibrinausfall in gleicher Weise.

Der Fibrinausfall im entstehenden und allmählich sich retrahierenden freien Gerinnsel, beispielsweise im Retraktionsröhrchen, bietet im Dunkelfeldmikroskop betrachtet ein völlig verschiedenes Bild und Geschehen: zu Beginn des Gerinnungsvorganges fällt zunächst die kompakte Fibrinmasse aus, die anfangs zwar eine Andeutung von Gerinnungsstreifen aufweist, in der Folge jedoch völlig strukturlos erscheint. Mit Fortschreiten des Gerinnungsprozesses und mit beginnender Retraktion erscheinen in der kompakten strukturlosen Fibrinmasse, auf derselben freiliegend oder von dieser teilweise oder gänzlich überdeckt, durch Aufbau durch Protein-



Abb. 2. Spitzendigende längliche Proteinkörnchen, reihenförmig angeordnet mit dichotomischer Verzweigung

Abb. 3. Gleiche Reihe wie Abb. 2 mit Zerfall der Proteinkörnchen zum Fibrinfaden führend.

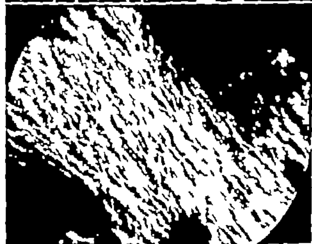
Abb. 4. Aus zerfallenden Proteinkörnchen entstehender Fibrinfaden

Abb. 5. Fibrinfäden größeren Kalibers aus reihenförmig angeordneten länglichen, spitzendigenden Proteinmassen entstehend mit einer dichotomischen Verzweigung

tropfen Fibrinfäden oder durch Proteinmassen Fibrinfibrillen verschiedenen Kalibers, die sodann bündelförmig zusammenrückend zu Fibrinsträngen sich einordnen und so die kompakte strukturlose Fibrinmasse nebst einzelnen Fibrillen weithin durchziehen.



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eingestellt, zeitlich verfolgt werden: zuerst ordnen sich die länglichen Proteinmassenteile zum ersten Längsrand ein und hernach teilweise quer überfließend zum zweiten, einen Längsleerraum dazwischen freilassend (Abb. 7, 8, 9).

Dieser Vorgang wiederholt sich in ununterbrochener Reihenfolge bis zur Bildung der langen Fibrille mit den zwei Längsrändern und dem dazwischen eingefaßten Längsleerraum (Abb. 10). In vielen Dunkelfeldaufnahmen finden sich die gebildeten Fibrillen auf der strukturlosen Fibrinkompaktmasse gelagert.

Bildung der Fibrillenstränge und ihr Verhalten zur kompakten, strukturlosen Fibrinmasse

1. *Bildung der Fibrillenstränge.* An die Phase des Aufbaues der Fibrinfibrillen schließt sich die Phase der Bildung der Fibrinstränge an, die durch bündelförmiges Zusammenrücken zahlreicher Fibrillen zur engen Strangbildung werden.

Nach unserer Auffassung wird dieses Phänomen durch die Wirkung des Hyalomers der Thrombozyten ausgelöst. Lichtoptisch läßt sich vermittelst des Deckglasverfahrens nachweisen, daß die im thrombozytenfreien Plasma ausgefallenen, regellos angeordneten Fibrinfäden bei Diffusion von Hyalomer vom Rande des Deckgläschens her sich zunächst parallel einstellen, um sodann zu bündelförmigen, schnurartigen Strängen zusammenzurücken (Abb. 11, 12, 13).

Der im freien Gerinnsel entstehende Fibrillenstrang setzt sich aus zahlreichen Fibrillen zusammen, deren Bündelung in hämolytisch verfärbtem Plasma deutlich zum Ausdruck kommt (Abb. 14). Zuweilen finden sich an einem Strangende zahlreiche bürstenartig auseinander strebende Fibrillen, auf diese Bündelung hindeutend (Abb. 15). In zahlreichen weitern Aufnahmen wird die Zusammensetzung der Stränge aus multiplen Fibrillen mit charakteristischem Längsleerraum dargestellt (Abb. 16).

2. *Verhalten einzelner Fibrillenstränge.* Ein Fibrillenstrang, die kompakte Fibrinmasse weithin durchziehend, kann am Rande der

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Abb. 11. Regellos angeordnete Fibrinfäden im Deckglaspräparat in thrombozytenfreiem Plasma.

Abb. 12. Paralleleinstellung der Fibrinfäden durch Einwirkung des Hyalomers.

Abb. 13. Schnurartige Fibrinstrangbildung durch bündelförmiges Zusammenrücken der Fibrinfibrille.



16 17 18

Abb 14 Fibrillenstrang aus hämolytisch verfärbtem Plasma 24 Stunden nach Auflösung der kompakten überdeckenden Fibrinmasse. Enge Bündelung der Fibrillen ersichtlich.
Abb 15. Ausgefranstes Ende eines hämolytisch verfärbten Fibrillenstranges. Besenreiserartiges Auseinanderstreben der Fibrillen.
Abb 16 Fibrillenstrang aus mehreren Fibrillen zusammengesetzt. Längsränder und Längsleerraum einer einzelnen Fibrille sichtbar
Abb 17 Austritt eines Fibrillenstranges aus der kompakten Fibrinmasse in den Leerraum des Blickfeldes einen Teil der Fibrinmasse mitreißend.
Abb 18 In den Leerraum des Blickfeldes aus der kompakten Fibrinmasse ausgetretener Fibrillenstrang ins Leere endigend.

selben in den Leerraum des Blickfeldes austreten und einen Teil der passiven Fibrinmasse mitreissen (Abb 17) Der Fibrillenstrang kann nach seinem Austritt aus der Fibrinmasse in den Leerraum gewissermassen blind endigen (Abb 18) oder an der Wand des Glasröhrchens fest ankleben, so das Fibringerinnsel daran fest fixierend. Diese Fixation kann nach beendeter Retraktion derart innig sein, daß es nicht ohne weiteres gelingt, den Strang von seiner Wandfixation abzutrennen und damit das Gesamtgerinnsel frei zu bekommen, was schließlich nur durch Durchschneiden des Stranges gelingt.

Wir werden auf dieses Verhalten bei der Besprechung des Retraktionsvorganges eingehend zurückkommen.

Zusammenfassend ergibt sich aus der Betrachtung des Werdeganges der Fibrinfibrillen, daß beim Gerinnungsvorgang zunächst die kompakte, strukturlose Fibrinmasse ausfällt und nachfolgend aus Proteinaufbau die Fibrinfäden und Fibrinfibrillen. Durch bündelförmiges Zusammenrücken der Fibrillen entstehen hernach die Fibrinstränge, welche die Fibrinmasse weithin durchziehen auf derselben freiliegend oder davon teilweise oder gänzlich überdeckt. Über ihr weiteres Verhalten und ihre Einwirkung auf die kompakte Fibrinmasse bei der Retraktion wird im folgenden Abschnitt berichtet.

Über die Fibrinquerstreifung beim Retraktionsvorgang

Querstreifungsbefunde im Fibrin wurden erstmals 1940 von Wolpers und Ruska im Liquor cerebrospinalis bei tuberkulöser Meningitis und später von mehreren Forschern elektronenmikroskopisch bei beschattetem Fibrin erhoben. So wies 1947 Wolpers bei mit Osmium beschattetem Fibrin labile Querstreifen nach. Morrison und Doppelt fanden 1954 bei fixiertem und beschattetem Fibrin eine Querstreifung mit einer Periodizität von 230 Å mit feinen intermediären Rändern. Kuhnke und Holze bestätigten 1957 diesen Befund bei Fibrin aus Rinderfibrinogen bei Thrombinzusatz ausgefallen. Hall und Slayter wiesen 1959 an beschatteten Segmenten der Fibrinfibrillen eine Querstreifung nach, durch Geradeausrichtung der Fibrinmoleküle bedingt. Die dunklen Bänder sollen den Terminalknötchen, das hellere Zwischenband den Zentralknötchen entsprechen.

1 Vergleich der Befunde von Querstreifung bei Myofibrillen und Fibrinfibrillen. Aus unseren Untersuchungen über die Retraktion des Fibringerinnsels mit dem Vorkommen der Querstreifung der Fibrinfibrillen geht retrospektiv hervor daß gewisse Analogien mit den Befunden und Vorgängen bei der Kontraktion der quergestreiften Muskelfaser bestehen, so daß zur Erleichterung des Verständnisses unserer Retraktionsbefunde sich ein kurzer zusammenfassender vergleichender Überblick zwischen beiden Vorgängen empfiehlt.

Die quergestreifte Muskelfaser weist im Inneren Pakete von winzigen vom Querschnitt eines Mikron betragenden, längsver

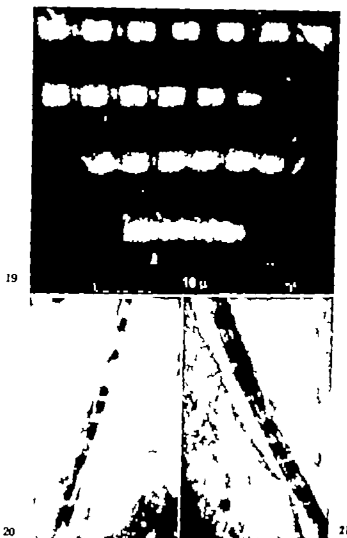


Abb. 19 Phasenkontrastaufnahmen einer Myofibrille im Verlauf einer Kontraktion. Verkürzung der Bänder I und Verschwinden der Zone H. (Aus «Endeavour» VI. XX mit Erlaubnis des Autors H. E. Huxley)

Abb. 20 Fibrinofibrille vor der Retraktion. Bänder I hell und unverkürzt. Bänder A dunkelgefärbt, gut abgegrenzt gegenüber Bänder I. Z-Linien nicht sichtbar wegen Überdeckung mit kompakter Fibrinmasse.

Abb. 21 Fibrinofibrille nach der Retraktion. Bänder I hell, verkürzt, Bänder A unverkürzt, dunkel gefärbt, Z-Linie nicht sichtbar wegen Überdeckung mit kompakter Fibrinmasse.

laufenden Myofibrillen auf, ähnlich wie die Fibrillenstränge sich aus Fibrinfibrillen zusammensetzen. Die Myofibrillen sind sehr eng aufeinander geschlossen, füllen den ganzen Querschnitt der Muskelfaser aus und erstrecken sich über deren ganze Länge, ähnlich wie die Fibrinfibrillen über den ganzen Strang. Die Myofibrillen stellen den kontraktilen Apparat der Muskelfaser in hochorganisierter Form dar. In ähnlicher Weise geht der Verlauf des Retraktionsvorganges der einzelnen Fibrinfibrille vor sich. Die Myofibrille weist eine Querstreifung, durch alternierende Bänder und Zonen bedingt, auf, dunkle und aufgehellte, mehr oder weniger dichten Bandregionen entsprechend. Die dunkle Region wird als Band A, die aufgehellte als Band I bezeichnet, das durch eine scharfe, dichte Linie entzweigeteilt ist, der Linie oder Membran Z, die quer durch die ganze Breite der Myofibrillen und durch deren Bänder verläuft. Das Band A wird durch die Zone H in zwei Hälften geteilt. Die quergestreifte Fibrinfibrille weist ähnliche Bänder auf: die Bänder A und I und die Linie Z, die quer durch den ganzen Querschnitt des Bandes I verläuft, die Ränder miteinbeziehend.

Eine Zone H im Band A konnten wir nie nachweisen. Im Verlauf der Kontraktion der Myofibrille konstatiert man in Phasenkontrastaufnahmen eine Verkürzung der Bänder I und das Verschwinden der Zone H bei Unverändertbleiben der Bänder A und der Linie Z (Abb. 19). Ähnlich verhalten sich die Bänder der quergestreiften Fibrinfibrille bei der Retraktion: Verkürzung der Bänder I bei Unverändertbleiben der Bänder A. Wegen Überlagerung durch die kompakte Fibrinmasse kommt die Linie Z nicht immer zur Darstellung, weder in der nichtretrahierten noch in der retrahierten Fibrille (Abb. 20, 21), während sie bei andern Aufnahmen scharf gekennzeichnet erscheint (Abb. 23, 24, 25, 27).

2. Vorkommen von Querstreifung bei Fibrillen des hämophilen Plasmas

Wie im Abschnitt über die Untersuchungsmethoden kurz erwähnt, wurden wir durch Befunde von Querstreifung bei Fibrillen von hämophilem Plasma auf dieses Vorkommen aufmerksam gemacht: durch den ganzen Querschnitt der Fibrille durchgehende helle, scharf gezeichnete Streifen mit Einbezug der Fibrillenränder, dazwischen trübe, kurze Bänder bei einer Fibrille im Magnesiumsulfatplasma; helle, sehr scharf gezeichnete Streifen (Abb. 22, 23).

Die Erfassung der Phase der Querstreifung bei der Retraktion der Fibrillen im hämophilen Plasma auf dessen verlangsamte Gerinnung zurückführend, bewog uns, Gerinnung und Retraktion von



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Abb 22. Fibrinfibrille aus hämophilem Plasma. Andeutung von Querstreifung.
Abb 23. Fibrinfibrille aus Magnesiumsulfatplasma einer hämophilen Überträgerin. Z Linien, dazwischen durchsichtige, aufgehellte Querbänder

normalem Plasma zu hemmen. Durch Zusatz von winzigen Mengen von Heparin gelang uns die gewollten Gerinnungs- und Retraktionsphasen zur Untersuchung zu erfassen. Später konnte die Gerinnungs- und Retraktionshemmung vorteilhafter durch Unterkühlung von normalem Blut und Plasma ohne Zusätze erzielt werden

Die Befunde von Querstreifung bei gerinnenden und sich retrahierenden Fibrinfibrillen haben uns veranlaßt ihre verschiedenen Phasen nach dem zeitlichen Vorkommen der Querstreifungserscheinungen einzuteilen in Fibrillen bei beginnender und solche bei fortschreitender bzw. vollendeter Retraktion.

3 Fibrillen mit Querstreifungsbefunden bei beginnender Retraktion. Zuweilen finden sich von kompakter Fibrinmasse leicht überdeckt, einzelne Fibrillen mit deutlich gezeichneter Z-Linie und dazwischen liegenden breiten, hellen Bändern (Abb. 24)

Aufschlußreicher sind Fibrillen mit fragmentartiger Einteilung in Rechtecke durch quere durchgehende Spaltbildungen durchtrennt, die Ränder miteinbeziehend. Diese Spaltbildungen dürften Z-Linien entsprechen (Abb 25) In der gleichen Aufnahme finden sich am unteren Fibrillenende stark verkürzte Fibrillenfragmente bei anscheinend verbreiterten Z-Linien Es hat den Anschein, als ob diese Fragmentverkürzungen und verbreiterungen einem teil-

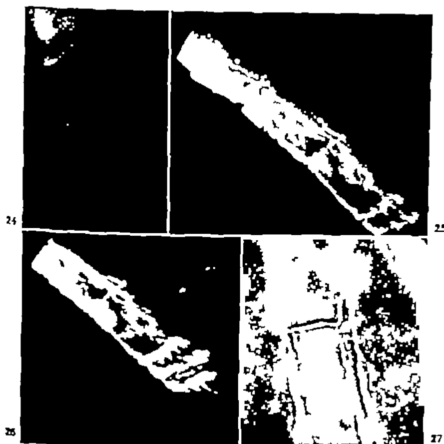


Abb. 24 Von kompakter Fibrinmasse leicht überdeckte Fibrille mit Z-Linien und dazwischen liegenden breiten Bändern.

Abb. 25 Fibrille mit fragmentartiger Einteilung in Rechtecke, durch quere durchgehende Spaltbildung getrennt

Abb. 26 Gleiche Fibrille von Abb. 25. Am untern Fibrillenende stark erkennbare Fibrillenfragmente mit anscheinend erbreiterten Z-Linien.

Abb. 27 Zwischen zwei Fibrillenfragmenten mit Einbezug der Ränder durchgehende Z Linie, eine gelenkartige Verbindung darstellend.

weisen Retraktionsvorgang am unteren Fragmentende entsprechen konnten (Abb 26) In einer weitern Aufnahme deutet die scharfe Wiedergabe der Z Linie mit Einbezug der Fibrillenränder auf eine gelenkartige Trennung zwischen oberem und unterem Fibrillenrechteck an (Abb 27) so daß sich uns die Vermutung aufdrängt ob die Z Linien nicht einer Trennung oder Abspaltung des Fibril



28 29 30

Abb. 28. Fibrillenstrang mit durchgehender Querstreifung.
Abb. 29. Fibrillenstrang mit durchgehender Querstreifung.
Abb. 30. Multiple quergestreifte Fibrillen, eng aneinander gelagert in quergestreiftem Fibrillenstrang, von kompakter Fibrinmasse teilweise überdeckt.

lenkerns beim Retraktionsvorgang entsprechen könnten, mit Einbezug der Fibrillenränder.

4. Fibrillen mit Querstreifungsbefunden bei fortschreitender oder vollendeter Retraktion. Aufnahmen von Fibrillensträngen weisen oft eine durch das ganze Fibrillenbündel durchgehende Querstreifung auf (Abb. 28, 29) bei scharfer Darstellung der Z-Linien. Bei der Aufnahme eines Fibrillenstranges ist der sehr seltene Befund eines Dutzend quergestreifter Fibrillen dargestellt, eng aneinander gekuppelt, von strukturloser Fibrinmasse leicht überlagert, welche die durch den ganzen Strang durchgehende Querstreifung erkennen lassen (Abb. 30).

Bei den meisten Aufnahmen von fortschreitender oder vollendeter Retraktion hat es sich erwiesen, daß die Fibrillenstränge

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Abb. 34. Eingekerbte Längsränder einer retrahierten Fibrille. Durchgehende Z-Linien?
Abb. 35. Unterer Teil der retrahierten Fibrille der Abb. 31. Verschwinden der Querstreifung bei totaler Aufquellung.
Abb. 36. Unterer Teil der retrahierten Fibrille der Abb. 33. Verschwinden der Querstreifung bei totaler Aufquellung.
Abb. 37. Zerfallende retrahierte Fibrille mit Arrosion der Längsränder und dazwischen liegenden, sich entleerendem Leerraum.

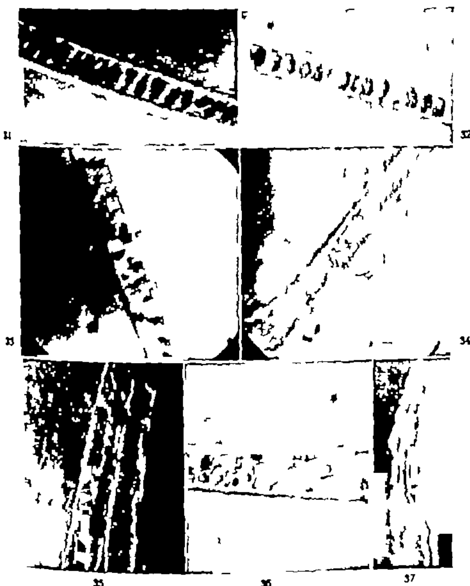


Abb. 31 Auf kompakter strukturloser Fibrinmasse gelagerte, retrahierte Fibrille in haemolysiertem Plasma. Darstellung der dunkel gefärbten nichtkontraktilen und der hellen, verkürzten, eng anliegenden kontraktilen Schichten mit z.T. angedeuteten Randeinkerbungen. Beide Schichten im Längsfaserraum der Fibrille.

Abb. 32. Dunkelgefärbte nichtkontraktile und helle, verkürzte kontraktile Schichten wie in Abb. 31 im Längsfaserraum enthalten.

Abb. 33. Retrahierte Fibrille aus leicht haemolysiertem Plasma. Nichtkontraktile Schichten nur fleckenförmig verfärbt. Ihre Massenanteil kommt gegenüber den hellen, verkürzten deutlich in Erscheinung. Beide im Längsfaserraum enthalten.

in der Regel in einzelne Fibrillen sich zerlegen, was die Darste der Auswirkung der Retraktionsvorgänge in denselben erhe erleichtert. Die einzelne Fibrille besteht nach vollendetem Au aus zwei Längsrändern und dazwischen dem Längsleerraum (10) Es hat sich nun erwiesen, daß der Retraktionsvorgang i Fibrillen mit Leerlängsraum stattfindet auf dessen Ränder (greifend, worauf durch deren Einkerbungen hingewiesen (Abb 31 32 33 34)

Im hämolysierten Plasma ausgefallene Fibrillen weisen sive dunkel gefärbte nicht kontraktile Schichten auf (Banc abwechselnd mit hellen, verkürzten, enganliegenden kontrak Schichten (Band I) beide im Längsleerraum bei leicht eingek ten Rändern (Abb. 31) In einer anderen Aufnahme einer Fib aus leichter hämolysiertem Plasma ausgefallen sind die nichtl traktilen Schichten nur fleckenförmig verfärbt trotzdem kor ihre Massivität gegenüber den hellen, verkürzten kontrakt Schichten deutlich zum Ausdruck (Abb 33) Bei einer weitern , nahme (Abb 34) kommt das Übergreifen der Querstreifung die Längsränder deutlich zum Ausdruck.

Die Beobachtung daß etwelche Zeit nach vollendeter traktion sich keine Querstreifungen mehr der Fibrillen darstel lassen und die Fibrillen dabei aufgequollen erscheinen, wird du den Nachweis erklärt, daß die Fibrillen nach vollendeter Retrakt an einem ihrer Enden der Querstreifung verlustig gegangen s bei gleichzeitigem Aufquellen des betreffenden Endanteiles (Al 35 36) Diese Erfahrung hat uns daher veranlaßt, die ausfallend Fibringerinnsel beim ersten Beginn der Retraktion dem Mik röhrchen zu entnehmen und unter Deckglas zu bringen, um (gewollte Retraktionsphase zu treffen. Nach einiger Zeit, zuweil nach mehreren Tagen, stellen sich in dem mit Canadabalsam ei gerahmten Deckglas befindlichen Nativpräparat Auflösungse scheinungen ein und es zerfallen die einzelnen Fibrillen, ei Arrosion der Längsränder und des Längsleerraumes aufweiser (Abb 37) Bei anderen Aufnahmen sind die Zerfallserscheinunge multipel (Aufquellung Tropfenauflösung völliger Zerfall usw

5 Vorkommen der Querstreifung im Magnesiumsulfatplasma Ir Deckglaspräparat eines Magnesiumsulfatplasmas, wie es zur Be urteilung der Morphologie und des Verhaltens der Thrombozyte vornehmlich bei Hämophilie verwendet wird beobachtet man zu



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Abb. 38. Magnesiumsulfatplasma. Eng zusammengerückte, hellaufleuchtende Fibrinteilchen, eine Querstreifung darstellend.

Abb. 39. Magnesiumsulfatplasma, Präparat der Abb. 38 nach 19 Stunden. Bogenförmige hellaufleuchtende, dünnste Fibrinteilchen, sehr eng aneinandergedrückt einen langen quergestreiften Fibrinfaden darstellend.

weilen (unter welchen Prämissen ist uns noch unbekannt) charakteristische Querstreifungsreihen.

Zu Beginn erscheinen längere Reihen von kleinsten, hellaufleuchtenden Fibrinteilchen z. T. mit dichotomischen Verzweigungen. Diese Teilchen verschmälern sich alsdann und rücken eng aneinander zusammen als hellaufleuchtende bogenförmige, quergestellte Teilchen eine Retraktionsreihe darstellend (Abb. 38). Nach längerem Zeitintervall, nach 19 Stunden, sind diese bogenförmigen, dünnsten Fibrinteilchen aufs engste aneinander zusammengerückt zu einem langen quergestreiften Fibrinfaden (Abb. 39).

In einer seltenen Aufnahme von hämophilem $MgSO_4$-Plasma fand sich eine Fibrille mit dem charakteristischen Anzeichen der Querstreifung. Merkwürdigerweise handelte es sich um das Plasma einer Hämophilieüberträgerin (Abb. 23).

Über die beim Fibrinausfall ausgeschiedenen Produkte und über ihr Verhalten bei der Retraktion

Überblickt man die Vorgänge beim Fibrinausfall und das Verhalten der Fibrillen beim Retraktionsvorgang, dann ergibt sich zusammenfassend, daß das Endprodukt der Blutgerinnung nicht

einheitlich ist. Das ausgefallene Fibrin besteht einerseits aus der kompakten Masse, die zu Beginn eine Andeutung von Gerinnungsstreifen aufweist, in der Folge jedoch strukturlos erscheint, und andererseits aus den durch Proteinaufbau entstehenden Fibrinfäden und Fibrinfibrillen.

Der Retraktionsvorgang spielt sich aktiv nur in den Fibrillen ab, die kompakte, strukturlose Fibrinmasse verhält sich dabei passiv und kann zuweilen von einem aus ihrem Zusammenhang in den Leerraum des Blickfeldes austretenden Fibrillenstrang teilweise mitgerissen werden (Abb. 17).

Die Fibrillen und namentlich die Fibrillenstränge auf der Fibrinmasse liegend oder davon teilweise oder gänzlich überdeckt, durchziehen weithin die Fibrinmasse. Zuweilen treten sie aus derselben in den Leerraum hinaus (Abb. 18), können dann ins Leere endigen oder an der Wand des Retraktionsröhrchens so fest ankleben, daß sie nur durch Abreissen oder Durchschneiden freizubekommen sind.

So ergibt sich, daß die die strukturlose, kompakte Fibrinmasse weithin durchziehenden Fibrillen oder Fibrillenstränge bei ihrer verkürzenden Retraktion die sich passiv verhaltenden Fibrinmassen mitreißen und so zur Gesamtretraktion des Fibringerinnsels führen. Dazu können aus der kompakten Fibrinmasse in den Leerraum austretende Fibrillenstränge sich an der rauhen Glaswand festsetzen, ankleben und so bei ihrer Retraktion die Fibrinmasse an sich ziehend das Gerinnsel in toto zur Retraktion bringen.

Zusammenfassung

1. Beschreibung des Proteinaufbaues der Fibrinfäden und Fibrinfibrillen und ihres bündelförmigen Zusammenrückens zu Fibrinsträngen, welche die strukturlose, kompakte Fibrinmasse weithin durchziehen.

2. Beschreibung der Retraktion mit der Querstreifung der Fibrinfibrillen. Der Retraktionsvorgang in der Fibrinfibrille ist demjenigen der Kontraktion der Myofibrille ähnlich, auch hier finden sich kontraktile und nichtkontraktile Schichten vor mit der bekannten Bändereinteilung.

3. Die beim Fibrinausfall entstehenden Produkte bestehen aus der strukturlosen, kompakten Fibrinmasse und den Fibrinfibrillen. Die Retraktion wird in den Fibrillen ausgelöst bei passivem Verhalten der kompakten Fibrinmasse.

Résumé

1. Description de la structure protéinique des filaments et fibrilles de fibrine et de leur rapprochement, rappelant des faisceaux sous forme de cordes de fibrine qui traversent les masses de fibrine compactes et sans autres structures.

2. Description de la rétraction avec striation des fibres de fibrine. La rétraction d'une fibre de fibrine ressemble à la contraction de la fibrine musculaire, dont l'alternat des bandes contractiles et rigides est connu.

3. Les produits formés lors de la précipitation de la fibrine sont composés de masses de fibrine homogènes et compactes et de fibrilles de fibrine. La rétraction est amorcée dans les fibrilles, alors que les masses compactes de fibrine restent passives.

Summary

1. The protein structure of fibrin threads and fibrils is described, together with their combination to form fibrin bundles or fibres which extensively traverse the amorphous, compact fibrin mass.

2. Retraction with the transverse stripes of the fibrils is described. This process is analogous with that of myofibril contraction, contractile and noncontractile layers being seen here too, with the well-known striated division.

3. The products of fibrin precipitation consist of the amorphous, compact fibrin mass and the fibrils. Retraction takes place in the fibrils, the mass remaining passive.

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Idiopathic Auto Immune Haemolytic Anaemia in Malaya

By Lie Injo Luan Eng and R. P Pillay

Auto-immune haemolytic anaemia is an acquired haemolytic anaemia due to the formation of antibodies in the patient directed against his own red blood cells, leading to increased haemolysis. In the majority of cases the aetiology of the disorder is unknown and is therefore designated idiopathic In others, however it is associated with certain diseases such as chronic lymphatic leukaemia or malignant conditions of the reticulo-endothelial system, disseminated lupus erythematosus e. a. and is referred to as symptomatic' The idiopathic as well as the symptomatic group can be subdivided according to the presence of a warm or cold antibody The warm antibody type is thought to be the more frequent one. Although the idiopathic type of haemolytic anaemia is thought not to be confined to any particular race or races, nearly all published cases were of European origin.

In this paper we present five cases of idiopathic auto-immune haemolytic anaemia of the cold antibody type in Asians. The young age group of these patients is also of interest, since the cold antibody type is usually found in elderly persons.

Methods

Haematological studies were carried out using standard methods.

Antibodies were studied by methods recommended by Dacie (1)

The presumptive tests for antibodies were carried out as described by Wintrobe (2)

The antiglobulin serum used was potent undiluted serum from the Commonwealth State Serum Institute.

The human γ-globulin used was kindly provided by the Lister Institute of Research, London.

Case Reports

Case 1 Azizah, Malay woman, 25 years old was admitted on February 28, 1962 to the General Hospital, Kuala Lumpur for anaemia. Two months prior to her hospital

admission in Kuala Lumpur she was admitted to the General Hospital at Ipoh for anaemia and jaundice of obscure origin. Since then she has always been pale. She denied having any illness prior to her attack of jaundice. On examination she was found to be pale with slight icteric tinge. The heart was slightly enlarged. The spleen as palpable 5 fingers and the liver 2 fingers below the costal margin. The blood picture on May 5, 1962 was as follows Hb 6.7 g% RBC 1.97 mil/cmm, PVC 21.0%, MCV 106.6 cμ, MCH 34.0 μμg, MCHC 3.10% WBC 5600. The differential count and platelet number were normal. Reticulocytes 21.6% There was slight poikilocytosis and definite anisocytosis. Spherocytosis was evident. Clumping of the red blood cells as noticed in the counting chamber which disappeared after warming. Fragility of the red blood cells to hypotonic saline solutions was increased. Haemoglobin analysis and alkali denaturation were normal. The indirect reacting bilirubin was increased. The direct Coombs test was positive. Indirect Coombs test was also positive with sensitization at 20 C as well as at 37 C. Presumptive tests for antibodies ga positive test for cold agglutinin. No other antibodies were found. Agglutination with normal red cells gave the following titres on May 11 1962 at 2 C 2048, at 20 C 32, at 25 C 8, at 37 C negative. With trypsin treated red cells higher titres were obtained at 20°C 8192, at 20 C 128, at 25 C 32 and at 37 C 2. Bloodgroup was A, Rh+ The bone marrow was hyperactive macro-normoblastic. Tests for G-6-pD Def., syphilis and lupus erythematosus were negative. No parasites were demonstrated in blood and faeces. The urine contained increased amount of urobilinogen but no bilirubin. X-ray of the chest and bones were normal. She was given one pint of packed red blood cells and the haemoglobin rose to 8.2 g% after the blood transfusion.

However it fell again to 7.3 g% four days later Treatment with prednisolone 40 mg daily was started on May 16. There was definite improvement in her condition following treatment. On May 26, the haemoglobin level was 11.4 g%, PCV 34.5%, reticulocytes 13.0%. Direct Coombs test was still positive, although slightly weaker than before treatment. It was of the non γ-type. Patient was discharged on May 26, 1962 and she did not come back for follow up.

Case 2: T C. H., 11 years old Chinese boy was admitted to the General Hospital on July 21 1962 with severe anaemia, jaundice and fever Two days prior to admission he voided dark red urine. The spleen and liver were enlarged, both about 2 fingers below the costal margin. His haemoglobin level was 2.8 g%. Blood smear were negative for malaria and he was treated with achromycin for his fever without success. He was given two pints of blood which gave only transient rise of the Hb level. On July 30, the following haematologic results were obtained. Hb 4.7 g%. RBC 1 43 mil/cmm, PCV 15%, MCV 103.4 cμ, MCH 32.4 μμg, MCHC 31.3%. WBC 5400/cmm with polymorphs predominating. There was slight thrombocytopenia. The red cells showed clumping in the counting chamber which disappeared after warming. There was anisocytosis and slight poikilocytosis. Spherocytosis was not conspicuous, normoblasts were seen in the peripheral blood. Direct Coombs' test was positive and was of the non γ-type. Fragility of the red blood cells to hypotonic saline solutions was not increased. Presumptive tests for antibodies showed strong cold agglutination. Tests for other anti-bodies were negative. Titration of the serum cold agglutinins with normal group 0 red cells gave the following titres at 4 C 512, at 20 C 32, at 37 C 2. With trypsinized red cells much higher titres were obtained. No haemolysin was detected in acidified or unacidified serum. Serum bilirubin was increased. Haemoglobin was of the normal A type. Alkali denaturation was within normal limits. Tests for G-6-pD Def. syphilis and lupus erythematosus were negative. Bone marrow was hyperplastic with reversal of the M.E ratio. He was given 30 mg prednisolone daily but despite this, his haemoglobin level dropped to 3.3 g%. The prednisolone dose was increased to 40 mg daily There was slight increase of haemoglobin over the next few days, but this was only of short duration. On August 9, the patient was extremely anaemic with haemoglobin level

3.1 g%. He was again given blood transfusion of packed cells, while the dose of prednisolone was increased to 60 mg daily. This was followed by a rise of the haemoglobin level, while the reticulocyte count decreased. On August 21 his haemoglobin was 7.6 g%. The agglutinin titres were lower than before treatment. Haemolysis was detectable at a titre 8 at 37 C, using normal serum as diluent. It was not tested at 20 C. When his haemoglobin was 10.6 g% and PCV 33.0% on August 28, the dose was gradually reduced. He was discharged on September 6. On September 21 his haemoglobin level reached 14.5 g%. However antibodies were still present although the reaction were weaker. The patient however developed a 'moon-face'. Prednisolone dosage was therefore reduced and finally discontinued on October 20. The haemoglobin level however dropped gradually and when it was 8.9 g% on November 8, prednisolone 10 mg daily was reinstituted. The haemoglobin level rose again. After another two months on the same dose, it was reduced and finally stopped. The haemoglobin dropped slightly but remained at about 13 g%, without reticulocytosis. Another check up 5 months after discontinuation of treatment, showed the haemoglobin level to be 12.9 g%. He appeared quite normal. Antibodies were however still present in the blood at low titre.

Case 3. Mrs. Lat., a Malay woman 38 years old, was admitted to the General Hospital, Kuala Lumpur on July 11 1962 with complaints of weakness and pallor. About three months prior to admission to the hospital, she had fever for one day and epistaxis. Pallor was then noted. She denied being ill shortly before this. Physical examination revealed nothing significant beyond pallor and slight jaundice. The spleen and liver were not enlarged. A complete blood study on August 2 revealed the following: Hb 7.1 g%, RBC 2.26 mil/cmm, PCV 22.1%, MCV 97.7 cμ, MCH 31.4 μμg, MCHC 32.1%. White blood cells and platelets normal. Reticulocyte count 5.7%. There was anisocytosis and slight poikilocytosis and polychromasia. Direct Coombs test was positive at dilution of 1:16. It was of the non γ-type. Presumptive tests showed the presence of strong cold agglutination. No other antibodies were demonstrated. Agglutinin titres were as follow: with normal blood group 0 cells at 4 C 512, at 23 C 32, at 37 C 2; with trypsin treated red cells at 2–4 C 2048, at 23 C 128, and at 37 C 8. The serum bilirubin was indirect and increased. The urine contained increased amounts of urobiline and urobilinogen, no bilirubin was found. Test for G-6-pD Def., syphilis and lupus erythematosus were negative. Haemoglobin analysis was normal, Malaria negative. The diagnosis of auto-immune haemolytic anaemia was made and the patient was given prednisolone 40 mg daily. There was dramatic response to treatment. The haemoglobin level rose rapidly. She was discharged on August 13 with a haemoglobin level of 11.0 g%, PCV 33.0%. On August 27 her haemoglobin was 12.1 g%, PCV 36.0%, and reticulocyte count was 3.2%. Antibodies were still present. Haemolysin was not present at 37 C. Titres of agglutinin using normal blood group 0 red cells were lower than before treatment: at 4 C 32, at 20 C negative, at 37 C negative; with trypsin treated red cells at 4 C 128, at 20 C 8, at 37 C negative. Prednisolone doses was reduced and finally stopped. The haemoglobin fluctuated between 11 and 12 g%, which was thought to be satisfactory. The direct Coomb's test was however still definitely positive and cold agglutinins were still present in significant amounts after 6 months discontinuation of the drug.

Case 4. Mrs. Rosm., Malay woman, 22 years old was admitted to the General Hospital, Kuala Lumpur on August 14 1962 because of severe pallor. There was no history of respiratory disease preceding her illness. On physical examination she was found to be very pale and slightly icteric. She had low-grade fever. Examination of the heart revealed systolic murmur audible over all areas. The spleen was palpable 2 fingers and the liver 1 finger below the costal margin. Haematological findings were as follows: Hb 2.5 g%, RBC 710,000/cmm, PCV 7.5%, MCV 105.6 cμ, MCH 33.2 μμg, MCHC 33.3 %. The red blood cells showed clumping at room temperature which did not entirely disappear at 37 C. There were circulating normoblasts. She was given

pint of packed cells the following day and at the start of transfusion, blood was again taken for full haematological study Hb 2.8 g%, RBC could not be estimated properly because of clumping of the red blood cells in the counting chamber PCV was 8.0%. Reticulocyte count 22.5%. Platelets 350,000/cmm, WBC 5000/cmm. Differential count showed shift to the left. Presumptive test for antibodies showed strong agglutination at 0 C. No other antibodies were demonstrated. Four days after transfusion her haemoglobin was found to be only 2.8 g%, while she looked more jaundiced. Apparently all the transfused red cells had been destroyed. She was given another pint of packed cells but now under cover of prednisolone 60 mg daily Haemoglobin rose to 4.1 g% after 4 days. Clumping of the red cells was still present. PCV was 12% reticulocytes 14%. Direct Coombs' test was still positive. It was of the non γ-type. Cold agglutinin titres with normal blood group 0 red cells were as follows at 4 C 1024 at 20 C 32, at 37 C 8, with trypsinised red cells higher titres were obtained at 4 C 8192, at 20 C 128 and at 37 C 32. Haemolysin was present at 37 C at titre of 8 when normal serum was used for dilution. It was not examined at 20 C. The haemoglobin rose to 5.7 g% on August 30. She was kept on high doses of prednisolone until the haemoglobin level reached 12.0 g%. The doses was then reduced. Unfortunately the patient left the hospital against medical advice, and we have not heard from her since.

Case 5: F J Ch., male Chinese, 35 years old, was admitted to the General Hospital Kuala Lumpur on August 14 1962 with severe jaundice and pallor Ten days prior to admission to the hospital he had sudden giddiness, weakness and passed dark urine. The attack was not proceeded by any respiratory disease or other infections. He had never had such an attack before. The patient appeared ill, pale and icteric. His spleen was just palpable, his liver was felt three fingers beneath the coastal margin. Haematological examination revealed the following: Hb 2.8 g%, RBC 0.79 mill/cmm, PCV 10.0%, MCV 126.6 cμ, MCH 35.5 μμg, MCHC 28.0% Reticulocytes 33.0%. WBC 37,500 with definite shift to the left. Normoblasts were seen in the peripheral blood. There as definite anisocytosis and slight poikilocytosis with clear spherocytosis of the red blood cells. Presumptive tests for antibodies showed strong cold agglutination. No other antibodies were demonstrated. The direct Coombs test was positive and was of the non γ-type. Cold agglutinin titres were as follows at 5 C 128, at 20 C 8, at 37 C negative with trypsin treated red cells at 5 C 128, at 20 C 32, at 37 C negative. Tests for syphilis, G-6-pD Def., and for L. E. cells were negative. There were no malarial parasites seen in the blood. He was given pint of blood and 60 mg prednisolone daily The response to treatment was clear cut. On August 27 Hb was 11.5 g% PCV 37%, RBC 3.30 mil/cmm, MCV 112 cμ, MCH 34.2 μμg, MCHC 30.5%. Reticulocyte count 14%. The direct Coombs test was still positive but was definitely weaker than before treatment. Also the cold agglutinin titre went down. However trypsin treated red cells were found to be weakly agglutinated at 37 C in undiluted serum. In the meantime he developed moon-face' He also experienced epigastric pains which were relieved by antacid therapy The prednisolone dosage was gradually reduced without ill effects. When the dosage was reduced from 20 mg to 15 mg the haemoglobin level fell. However by maintaining the dosage at 20 mg, the haemoglobin level rose again, and was found to be 15.2 g on October 22, 1962. The prednisolone dosage was then reduced and finally stopped. His haemoglobin level has remained high without any therapy Five months after discontinuation of prednisolone therapy the haemoglobin was 15 g%. The direct Coombs test was however still weakly positive and low titre of cold agglutinin was still present.

Discussion

All five patients were cases of haemolytic anaemia with positive Coombs test. In addition to this, the presence of cold agglutinins

was shown in all five cases. Although the agglutinin titre at 4° C was not high in most of them, it was certainly above those usually found in normal sera. In all cases the cold agglutinin titre was much higher when trypsin treated red cells were used. Further the thermal range of activity was high extending even to 37° C in several of the cases. The diagnosis of acquired haemolytic anaemia due to auto-immune antibodies can be made with certainty in all five cases. The presence of cold agglutinins whose activity was greatly enhanced by lowering the temperature and which were found to be of the non γ-globulin type, makes them belong to the cold antibody type of auto-immune haemolytic anaemia. There are however several unusual features concerning these cases. The titre of cold agglutinins was in general relatively low while the thermal range of activity was high. In all patients, agglutination was apparent at tropical room temperature (28° C to 30° C) in the counting chamber. Agglutination was even demonstrated at 37° C. In addition to this, haemolysins could be demonstrated at 37° C at a low titre using trypsinized red cells in several of the patients. The presence of this haemolysin was not shown in the presumptive tests in which the patients own red cells were incubated in their respective sera.

The serological features in our cases resemble in certain ways those described by Dacie (3) as 'less typical cases of haemolytic anaemia of Cold-Antibody type, distinct from the Cold Haemagglutinin Diseases. As was also the case in Dacie's patients, none of our patients experienced Raynaud's phenomena and for this reason they cannot be classified as suffering from typical cold-haemagglutinin disease. Since no definite disease could be demonstrated to be associated with the haemolytic condition, we have designated them idiopathic. Of interest is the young age of our patients. The youngest was 11 years old. In Europe it is well known that auto-immune haemolytic anaemia of the cold antibody type occurs following virus pneumonia. No respiratory infection preceeded the haemolytic condition in any of our patients. The possibility of other unknown virus infections as possible aetiological factors should be considered. However as a complication, haemolysis usually follows the viral disease and is often found during convalescence. In addition the haemolytic episode complicating virus infections is usually transient. In our patients haemolytic symptoms appeared from the onset. Case 1 and 3 came for consultation after

suffering from haemolytic anaemia for many months. In cases 2, 3 and 5 who came regularly for follow up, it was shown that the haemolytic condition was controlled only with prednisolone over a prolonged period. Cases 2 and 4 who responded dramatically to prednisolone treatment could not be followed up.

It is possible that this picture, which is rather atypical for European countries is the usual picture found in the tropics. Data for this area of the world however is very scanty. One of the authors (LIE INJO) has found three cases of idiopathic auto-immune haemolytic anaemia of the cold antibody type in Indonesia, one in a Chinese man and two in Indonesian women. Two of these cases have been demonstrated at clinical meetings in 1956. None of those cases were older than 40 years, and their serologic findings were very similar to those described in this paper. Unfortunately those cases were not published because their clinical data were lost in the hospital's recording room. THURAISINGHAM demonstrated a case of idiopathic auto-immune haemolytic anaemia of the cold antibody type in a young woman from Penang at a clinical meeting in 1961. It is probable that this disease is not at all rare in the tropics.

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Summary

Five cases of idiopathic auto-immune haemolytic anaemia of the cold antibody type are described. Three were Malays and two were Chinese. They ranged in age from 11 years to 38 years. None of them experienced Raynaud's phenomenon, and for this reason they cannot be classified as suffering from typical cold haemagglutinin disease. Serological findings are described. All cases responded to prednisolone treatment.

Résumé

Rapport de 5 malades (3 malais et 2 chinois), agés de 11 à 38 ans, atteints d'une anémie idiopathique hémolytique auto-immunologique provoquée par des anticorps à froid. Comme aucun de ces cas ne présentait le phénomène de Raynaud, une maladie classique d'hémagglutinine à froid pouvait être exclue. Description des résultats sérologiques. Tous les cas répondaient bien au traitement par le prednisolone.

Zusammenfassung

Es wird über 5 Patienten (3 Malayen und 2 Chinesen) im Alter von 11 bis 38 Jahren mit idiopathischer autoimmun-hämolytischer Anämie vom Kälteantikörpertyp berichtet. Da keiner dieser Fälle ein Raynaudsches Phänomen aufwies, lag keine typische Kälteagglutinin-Krankheit vor. Die serologischen Befunde werden beschrieben. Alle Fälle sprachen auf Prednisolonbehandlung an.

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The Action in vitro and in vivo of Sodium Versenate on the Phagocytic Activity of Neutrophile Leukoc

By O Forssman and P Nordqvist

The use of chelating agents in medicine has increased n t last few years (Review 1) However very little is known about th ir damaging effects, if any upon living cells. In vitro experim nts have conclusively shown that Na_2 EDTA disaggregate clump of leukocytes obtained by adding saline to blood (2) This paper reports the influence of Na_2 EDTA upon the phagocytic property of neutrophile leukocytes. It will be shown that Na_2 EDTA prevents the uptake of iron powder by this kind of leukocytes suspended in plasma. However the same dosage of Na_2 EDTA (mg per ml plasma) administered intravenously during 4 hours does not exert this influence on the neutrophiles.

Methods

In Vitro Experiments

80 ml heparinized blood was draw from healthy person and allowed to settle for 2 hours. The plasma layer was removed and centrifuged for 4 minutes at 1000 rpm. The supernatant was discarded. 10 ml saline as added to the bottom layer consisting mainly of white blood cells. After mixture of cells and saline the tube was allowed to stand in vertical position for 10 minutes. During this time macroscopic aggregates were formed which spontaneously settled to the bottom. Microscopically the sediment contained large clumps of granulocytes while the supernatant as composed partly by single cells, mainly neutrophile leukocytes, and partly by fairly small ggregates of the same kind of cells. Since the arious compositions of the tw layers might reflect arious properties of the granulocytes, the two layers were studied separately

Bottom layer One part of the aggregated leukocytes from the bottom of the tube was resuspended in 10 ml of the patient own plasma. Another part was disaggregated with 0.8 ml 1% solution of Na_2 EDTA and 10 ml of the same plasma was added. Admixture of 20 mg carbonyl iron powder SF (Fine Dyestuffs & Chemical Limited, Manchester; main particle size 1 4 μ) in both tubes. Thereafter they were kept at 37 °C for 30 minutes under gentle shaking at 5 minutes intervals



Fig 1 Aggregated cells from th
aggregates from the supernatant (
Practicall all cells are totally fill

Supernatant. Tw equal parts of the supernatant were centrifugated 1000 rpm for 4 minutes. T one of the sediments 0.4 or 0.8 ml N EDTA as added. Both tubes were then treated with plasma and iron powder as described bov

In Vivo Experiments

According to the technic recommended by Johnson 3 3 g Na EDTA as diluted ith 500 ml saline and given as Infusion during 4 hours. Befor and immediately after the infusion 80 ml blood were drawn and allowed to settle f hours 10 ml of plasma layer just abov the settled red cells were drawn. This plasma sa ple hich rich of leukocytes was mixed with iron powder and treated as describ d

Results

In vitro experiments. 11 experiments were performed and all showed the same tendency Moreover there were no prir l divergences between the results obtained from the bottom l er and from the supernatant.

When versenate was present in the samples there were no aggregates visible and the rate of phagocytosis by the leukocytes was very low or none. This was in sharp contrast ith observations made in the tubes not containing versenate. Regardless of the rate of aggregation—bottom layer or supernatant—there was a marked predominance of iron containing cells (fig 1)

In vivo experiments As expected the neutrophile in the samples drawn before the intravenous administration of Na_2 EDTA presented maximal phagocytosis. The treatment with Na_2 EDTA, however did not at all affect the phagocytic ability of human leukocyte suspension in plasma.

Discussion

The viability of the neutrophile leukocyte is among others estimated by its capacity to show phagocytosis. Our results showed a very decreased or abolished rate of phagocytosis in the presence of versenate. This finding has previously been mentioned by KUPER et al. (4) and recently GREENDYKE et al (5) found that human leukocytes suspended in plasma-saline mixtures showed inability to phagocyte opsonified erythrocytes in the presence of 0 4 mg per ml of K_2 EDTA, but were able to do so at levels of 0.2 mg per ml.

Our results show further that the phagocytosis in vitro was not arrested by cell clumping This may well be explained by the findings that mature granulocytes ith great surface charge have a low surface tension, i.e. a great phagocytic ability (6 7) The

action of versenate in this respect remains obscure. However it is known that other activities of the granulocyte membrane also are influenced by treating blood with a chelating resin in order to remove divalent cations. Thus, the adhesiveness of the human polymorphonuclear neutrophiles is lost and at the same time also the ability of these cells to migrate (8). In this connection it will be mentioned that phagocytosis will not occur in the absence of free calcium ions (9) and, furthermore, that plasma passed over an ion exchange resin to remove divalent cations, partially or completely restored its erythrophagocytotic capacity after re-addition of Mg^{++} and/or Ca^{++} (5).

The results obtained in vitro raise the question whether Na_2 EDTA used e.g. in the treatment of hypercalcemia and digitalis intoxication will exert the same damaging effect in vivo. In the clinical series 3 g Na_2 EDTA was slowly administered intravenously, i.e. in a dosage corresponding to that used in vitro. The most important finding to emerge from this investigation was that the phagocytic capacity of the neutrophils was *still* retained after the injection.

Summary

In vitro, the viability of granulocytes as measured by the capacity of digesting carbonyl iron is inhibited by disodium versenate. Most probably this is a function of an altered cell membrane potential, since at the same time artificially induced clumping of the granulocytes was lowered. The same dose (mg Na_2 EDTA/ml plasma) sometimes used clinically does not influence the phagocytic capacity of the granulocytes when given intravenously as infusion.

Résumé

La vitalité des granulocytes, mesurée par la capacité de résorber le carbonyl de fer est diminuée in vitro par le versenate de sodium. Cette diminution est probablement due à une modification du potentiel de la membrane cellulaire parce que une agglutination artificielle est parallèlement diminuée. La dose (mg Na_2EDTA/ml plasma) qu'on utilise parfois en clinique, n'influence pas le pouvoir phagocytaire des granulocytes lors d'une infusion intraveineuse.

Zusammenfassung

Die Lebensfähigkeit der Granulozyten, gemessen an ihrer Fähigkeit zur Aufnahme von Eisencarbonyl, wird in vitro durch Natriumversenat herabgesetzt. Dies ist wahrscheinlich auf eine Veränderung des Membranpotentials der Zelle zurückzuführen, da gleichzeitig die künstlich erzeugte Verklumpung der Granulozyten sich vermindert. Die gleiche Dosis (mg Na_2 EDTA/ml Plasma) wie sie gelegentlich klinisch verwendet wird, beeinflußt bei intravenöser Infusion die Phagozytosefähigkeit der Granulozyten nicht.

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Aus der Universitäts-Kinderklinik Heidelberg (Direktor: Prof. Ph. Bamberger)

Zytochemische Untersuchung von Zytoplasmafragmenten und Gumprechtschen Schollen bei akuten Leukosen *

Von Michael Hertl

Die Individualität des einzelnen Leukosefalles drückt sich sowohl in der Eigenart der klinischen Auswirkung der Markinsuffizienz wie in Besonderheiten des pathologischen Zellmaterials aus. Vor allem Naegeli und Rohr haben auf diese individuelle Prägung der Leukosezellen aus morphologischer Sicht unter Verwendung der Pappenheim-Färbung schon vor Jahrzehnten hingewiesen.

Nun wurden in den letzten anderthalb Jahrzehnten in steigendem Maße zytochemische Methoden für die Untersuchung der Leukosezellen herangezogen. Sie brachten nicht nur eine Bestätigung dieser Ansicht, sondern auch eine Vertiefung. Es zeigte sich nämlich, daß einige der zytochemischen Einzeleigenschaften der Zellorganellen isoliert ausfallen können, ohne daß dies schon aus dem Färbeergebnis nach Pappenheim zu vermuten wäre. So ergibt sich bei zusätzlicher Auswertung der zytochemischen Einzelergebnisse über die bekannte Polymorphie der Leukosezellen hinaus eine weitere Aufgliederung hinsichtlich der individuellen Prägung des Leukosezellmaterials. Früher haben wir darüber schon ausführlich berichtet.

Einen zweiten Vorteil bringt die zytochemische Methodik mit sich. Es ist die Möglichkeit, bei morphologisch gleich erscheinenden Gebilden durch Feststellung der einzelnen zytochemischen Eigenschaften die Identität zu beweisen oder auszuschließen. So finden sich bei vielen Leukosefällen, beim Einzelfall mehr oder weniger kernlose Zellteile: Zytoplasmafragmente, für die nach dem Pappen-

* Herrn Prof. Dr. Ph. Bamberger zum 65. Geburtstag.

HEIM-Bild die Einstufung als Thrombozyt bzw. atypischer Thrombozyt oder als Zellteil anderer Abkunft offen bleibt. Über zytochemische Untersuchungen zu dieser Frage soll im folgenden berichtet werden.

Ein dritter Vorteil, den die zytochemische Methodik vermittelt, ist weniger für die hämatologisch klinische Diagnostik sondern vielmehr für die funktionelle Pathologie der Leukosezellen und darüber hinaus für die allgemeine Zytologie von Bedeutung. Aus dieser Sicht werden zytochemische Befunde an GUMPRECHTschen Zellschatten mitgeteilt, die neue Aspekte zum Kernbau der Leukosezelle und der menschlichen und tierischen Zelle schlechthin erlauben.

1 Zytochemische Untersuchung, Beurteilung und Zuordnung von Zytoplasmafragmenten. Bei vielen Leukosefällen werden kleine basophile Gebilde gefunden, die in ihrer färberischen Eigenheit und Polymorphie schwer beurteilbar erscheinen und meist nicht beachtet werden. Sie finden sich von der Größenordnung kleiner Thrombozyten bis zur Größe der Erythrozyten und sind gelegentlich rund, oval, meist aber polygonal unregelmäßig gestaltet. Bei manchen Fällen werden sie nur als einheitlich schwach-basophil gefärbte zarte Gebilde gefunden, bei anderen enthalten sie einige bis zahlreiche eosinophile und/oder basophile, mittelgroße, gut sichtbare Granula. So liegt es nahe, sie für Thrombozyten, mehr noch für atypische Thrombozyten zu halten.

Bekanntlich ist die aplastische Thrombozytopenie ein Kardinalsymptom der Leukose. Megakaryozyten fehlen im Mark oder sind nur sehr selten anzutreffen. Die Thrombozytenzählung ergibt für die Blutperipherie daher nur sehr geringe Werte. Bei manchen Leukosefällen muß sich also eine merkwürdige Diskrepanz dieser Werte zu den offenbar zahlreicheren thrombozytenähnlichen Zytoplasmafragmenten im Knochenmarkausstrich ergeben. Wie geschildert ist aber ein großer Teil dieser Zytoplasmafragmente normalen Thrombozyten unähnlich, und er wäre dem Thrombozyten nur dann überzeugend zuordnungsfähig, wenn man für die akute Leukose die Möglichkeit einer pathologischen Thrombozytenmorphe annimmt. QUABER hat diese Frage bejaht und über seine Befunde berichtet.

Neben der PAPPENHEIM-Färbung wandten wir folgende zytochemische Untersuchungsmethoden am Ausstrich sowohl von normalem als auch leukotischem Knochenmark an. Für Aminosäuren: Tetrazolium-Reaktion (DANIELLI), Fastgreen-Färbung

Tabelle I

Zytochemie der Zytoplasmafragmente im Vergleich zu Thrombozyten, sowie zum Zytoplasma Paramyeloblasten und Parapromyelozyten III (zur Definition der Leukosezellen siehe Huth, 1 1962, 1963)

Nachweis von	Basophile Zytoplasmafragmente Typ I	Typ II Grundpl.	Typ II bas. Granula	Thrombozyten Grundpl.	Thrombozyten Granula	Para-Myeloblast Grundpl.	Para-Promyel. Grundpl.	bas. G
Basophilie (Pappenheim-Präp.)	+	+	++	(+)	O—+	+	+	+
Aminosäuren	+	+	++	(+)	+	+	+	+
Bas. Proteine	++	++	++	(+)	(+)	++	++	-
SH-Gruppen	O	O	+	O	O	O	O	+
DNS	O	O	O	O	O	O	O	O
RNS	++	++	++	O	(+)	++	++	+
Glykogen	O	O	O	+	O	O	O	O
Lipide	(+)	O—(+)	++	O	O (?)	(+)	O—(+)	-
Plasmalogene	(+)	(+)	+	O	O	(+)	(+)	-
Phospholipoide	O	O	+	O	O	O	O	+
Peroxydase	O	O	+	O	O	O	O	

Grundpl. = Grundplasma (lichtoptisch sichtbares Zytoplasma außerhalb der Granula).

bei pH 4,1 (Gedigk). Für Nukleinsäuren: Nukleal-Reaktion (Feulgen und Rossenbeck), Gallocyanin-Chromalaun, Toluidinblau und Methylgrün-Pyronin. Für SH- und SS-Gruppen: Reaktion nach Chèvremont und Frédéric. Für Kohlenhydrate: PAS-Reaktion nach Hotchkiss, auch nach Ptyalin-Einwirkung; Bestsche Karminfärbung. Für Lipide: Sudan-Schwarz B, Plasmal-Reaktion (Feulgen und Voit), Nilblau-Reaktion (Menschik). Von Enzymbestimmungen nur die Peroxydase-Reaktion (Sato), da bei der akuten Leukose die Bestimmung der alkalischen Phosphatase und der unspezifischen Esterase ohne Bedeutung ist.

Das Ergebnis der zytochemischen Bausteinanalyse ist in Tabelle I zusammengefaßt. Als Typ I bezeichnen wir dabei die basophilen Zytoplasmafragmente ohne Granula, als Typ II solche mit basophiler Granulation. Der Vergleich mit Thrombozyten deckt neben den geschilderten morphologischen Unterschieden auch markante zytochemische Unterschiede auf. Thrombozyten sind wesentlich ärmer an basischem Protein. Ribonukleinsäuren, Lipide, SH-Gruppen und die Peroxydase sind nicht nachweisbar. Dagegen ist im Thrombozyten eine geringe Menge von Glykogen zu finden. Um Thrombozyten, auch um pathologische Thrombozyten kann es sich also bei den diskutierten Zytoplasmafragmenten nicht handeln.

Die Eigenschaften der Zytoplasmafragmente bei der Färbung nach Pappenheim erweisen sich als identisch mit denen des Zytoplasmas der Leukozellen. Der Vergleich der zytochemischen Befunde ergibt tatsächlich Übereinstimmung insofern, als ein Teil der Zytoplasmafragmente von Paramyeloblasten, der andere von

Para Promyelozyten stammen muß. Hervorhebenswert ist die Bestimmung der Peroxydase deren positiver Ausfall bei basophil granulierten Zytoplasmafragmenten schon für sich allein genommen die Abgrenzung von Thrombozyten entscheidet.

Der letzte Beweis für diese Folgerungen ergibt sich wiederum durch morphologische Befunde. Bei intensivem Suchen sind gelegentlich akute Leukosezellen zu finden von denen Zytoplasmafortsätze pseudopodienartig weit vorgestreckt sind und den Anschein machen als sollten sie in Kürze abgeschnürt werden (Abb. 1 2)



Abb. 1 Entstehung von Zytoplasmafragmenten. Pseudopodien von Paramyeloblasten mit Abtrennung einzelner Füßchen, die sodann für typische oder atypische Thrombozyten gehalten werden können. Pappenheim-Färbung 3000 : 1

Die Abschnürung von Zytoplasmafragmenten ist jedoch nicht auf das Krankheitsbild der Leukose beschränkt, sondern auch im normalen Mark gelegentlich zu finden. Schulten beschreibt Zytoplasmafragmente als Abkömmlinge vom Zytoplasma des Promyelozyten und des Myelozyten. Das häufige, ja gelegentlich massenhafte Auftreten ist für Leukosefälle jedoch charakteristisch und muß mit der Zellabartung zusammenhängen. Die Lagerungsart und -dichte im Ausstrich läßt vermuten, daß die Abtrennung fast immer schon in vivo geschieht. Im Knochenmarkschnitt haben wir sie nicht gesehen, was aber nicht dagegen spricht. Höchstens parazellulär liegende Fragmente und einzelne im Ausstrich fixierte Abschnürungsvorgänge konnten erst nach der Entnahme des Knochenmarks entstanden und somit Ausdruck der Nekrobiose sein.

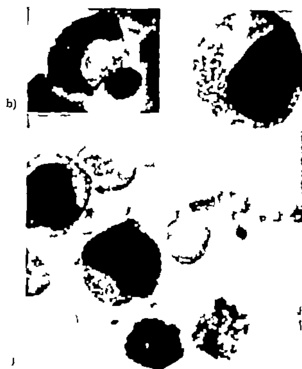


Abb. 2. Zytoplasmafahnen, demonstriert an Zellen der subakuten Leukose.) Morphologisch kaum auffällige Para-Promyelozyten und Para-Myelozyten. Unten eine granulierte Zytoplasmafahne mit den Eigenschaften des Promyelozyten-Zytoplasma. b) Abschnürung einer basophil granulierten Zytoplasmafahne bei einem Para-Metamyelozyten. PAPPENHEIM-Farbung. 1500 : 1

? *Beobachtungen an* GUMPRECHT*schen Kernschatten* GUMPRECHTsche Kernschatten sind gewöhnlich kaum näher beachtete Artefakte, die beim Ausstreichen der Zellen auf dem Objektträger entstehen. Es werden immer junge, unreife Zellen davon betroffen folglich muß die relativ lockere Struktur und der höhere Wasserreichtum der Zellen eine Ursache sein. Die Lagerungsart des Chromatins in der GUMPRECHTschen Scholle ist offenbar abhängig von der Intensität des Quetschdruckes und von der in vivo gegebenen Chromatinlagerung die wie am fixierten Präparat von feinkörnig bis feinfädig bis grobschollig verschiedene Übergangsstufen aufweist

Im gesunden Knochenmark ist die leichte Laeherbarkeit der Reticulumzellen geläufig. Bei Blutkrankheiten sind es vor allem die Zellen der lymphatischen und akuten myeloischen Leukosen, sowie der malignen Reticulose

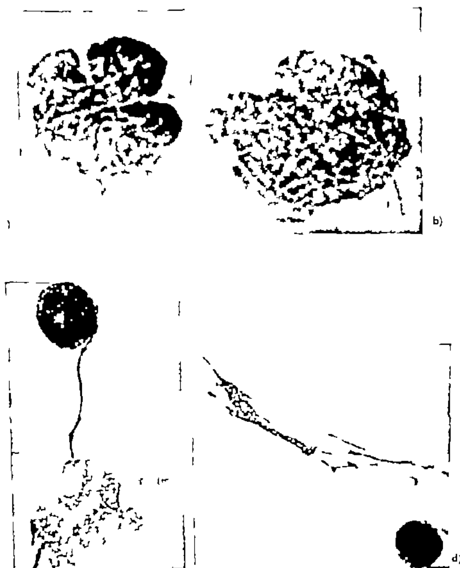


Abb. 3. Gumprechtsche Kernschatten. Akute (Paraleukoblasten- Para-Myeloblasten-) Leukose. Toluidinblau-Färbung.) und b) Leicht gequetschte Zellen mit der charakteristischen strähnigen Chromatinlagerung. b) An dieser Zelle ist die Kernmembran aufgerissen. Chromatinfäden sind ausgetreten.) Sehr stark ausgebreitetes Chromatin, das aus einheitlich großen DNS-Körpern besteht. Oben intakte Zelle mit etwa gleicher Chromatin-Korngröße. d) Gruppe von Chromatinfäden. Teil eines stark zerstörten und [illegible] Zellkerns. Intakter Paraoblast zum Vergleich.

Wir untersuchten mit Nukleotid Reaktionen die GUMPRECHTschen Schollen bei Fällen mit akuter Leukose und hatten dabei folgende Fragestellung Unter welcher struktureller Ordnung liegt das feulgenpositive Chromatin des Interphasenkernes vor und in welcher Beziehung steht es zu den kompakten Chromosomen des Mitosekernes?

Als Methoden benutzten wir neben der PAPPENHEIM Färbung, neben Galloxyanin Chromalaun und Methylgrün Pyronin insbesondere die FEULGEN Reaktion und die Toluidinblau Färbung deren scharfe Zeichnung für die morphologische Beobachtung besonders günstig war

Die färberischen Ergebnisse seien zuerst morphologisch interpretiert Folgende Lagerungstypen des Kernchromatins der GUMPRECHTschen Schollen sind abzutrennen

1 Das Chromatin ist zu einer ungegliederten, homogenen polygonalen Fläche auseinander geflossen Es ist keine Struktur mehr zu erkennen

2 Das sonst irregulär körnig oder fädig gelagerte Chromatin ist arealweise zu einer strähnigen Anordnung gelangt Offenbar haben die Zellen beim Ausstreichen nur eine leichte Schubwirkung erfahren.

3 Trifft eine besonders starke Quetschwirkung senkrecht zum Objektträger auf die Zelle platzen die Kerne offenbar auseinander Es zeigt sich irreguläre feinfädige netzige Struktur Die Netzfäden sind meist nicht gleichmäßig dick und an Überkreuzungsstellen häufig knotig aufgetrieben.

4 Übergänge von der 3 Lagerungsart führen zu dieser 4 welche die aufschlußreichste ist. Der hierbei wirksame Schub führt nicht nur zur strähnigen oder feinstreifigen Chromatinausrichtung sondern auch zum Aufreißen der Kernmembran Chromatinfäden treten aus der ursprünglichen Kernbegrenzung aus. Sie sind kurz und reichen etwa 40–80 μ weit Bei stärkster Vergrößerung erinnern sie an Perlschnüre wie auf einem gerade sichtbaren Faden aufgereiht folgt in kürzesten Abständen ein etwa gleich großes Chromatinpartikel dem anderen Meist liegen mehrere Perlschnüre nebeneinander Ihre Zahl ist vollkommen unregelmäßig, oft ungerade Die Lagerung ist immer unpaarig Nicht selten sind auch einzelne Schnüre zu finden. Diese können übrigens, von der Ausstreichrichtung geführt weite Präparatteile durchlaufen und

dabei über andere Zellen hinweg, an andere angelagert vorbeifüh ren. So sind sie unter Umständen bis 10 mm Länge verfolgbar

Zytochemisch teilen die Chromatinkörper dieser »Perl schnüre« die Eigenschaften der der Kernmembran angelagerten Chromatinteilchen einer intakten Zelle. Sie sind feulgenpositiv Auch die Verbindungsfäden geben die Reaktion auf Desoxyribo- nukleinsäure.

Die geschilderten, durch die Schubwirkung beim Ausstreichen gegebenen Auslagerungen aus dem Kernareal sprechen dafür daß die einzelnen Chromosomenpartikel untereinander fädig verbunden sind und es spricht nichts dagegen, diese Ordnung auch für die intakte Zelle anzunehmen. So bietet sich die bildhafte Vorstellung an, als ließen sich Chromatinperlschnüre wie Fäden von einem Knäuel abspulen.

Die Zytologie arbeitet bekanntlich mit der Hypothese daß die während der Mitose kompakten kurzen, färberisch kräftig dar stellbaren Chromosomen auch im Interphasenkern als Individuen, wenn auch in starkst entspiralisiertem Zustand vorliegen. Unsere Befunde an den Gumprechtschen Schollen stützen ohne Zweifel diese Vorstellungen, *ja stellen eine Beweismöglichkeit dar* wie sie am Interphasenkern kaum besser gewonnen werden kann. Hervor zuheben ist aber daß die Chromatinschnüre nicht paarig vorliegen was man aus der Vorstellung parallel liegender Chromosomen- paarlinge erwarten möchte

Weitere Fragen sind in diesem Zusammenhang noch offen Sind die Chromatinbrocken tatsächlich wie Perlen einer Kette auf einen Faden aufgereiht oder sind die Chromatinbrocken in ein elastisches, strumpfartiges Gebilde in Abständen eingeschlossen? Die nähere Klärung wäre für die Frage von Bedeutung, wie man sich den Stoffstrom vom Nucleolus zur Kernmembran, in einer Leit rohre oder nicht, vorzustellen hat. Ferner stehen nähere, gesicherte Kenntnisse über den Zusammenhang des feulgenpositiven Chro- matins mit dem feulgennegativen Nucleolus noch aus und es wäre möglich, daß auch dazu bestimmte Zonen der Gumprechtschen Schollen wesentliche Hinweise geben können.

Zusammenfassung

Es wird über die zytochemische und morphologische Beurteilung zweier Eigen- heiten von Leukosezellen berichtet. Die Zytoplasmafragmente, die bei der Pappenheim- Färbung nicht selten gefunden und möglicherweise als pathologische Thrombozyten

beurteilt werden, erweisen sich als Teil der Paramyeloblasten und Parapromyelocyten. An Gumprechtschen Schollen läßt die perlschnurartige Gestalt des beim Ausstreichen verschleppten Chromatins annehmen, daß die Chromosomen auch im Interphasenkern als Individuen wenn auch in starkst entspiralisiertem Zustand vorliegen.

Summary

The cytochemical and morphological evaluation of two characteristic features of leukaemic cells is reported. The cytoplasm fragments frequently found in Pappenheim staining and sometimes regarded as pathological thrombocytes appear to be parts of paramyeloblasts and parapromyelocytes. In Gumprecht shadows, the pearl-necklace effect of the chromatin produced in obtaining the smear suggests that the chromosomes are also present as individuals in the interphase nuclei though in largely uncoiled state.

Résumé

Rapport sur l'appréciation cytochimique et morphologique de deux particularités des cellules leucémiques. Les fragments cytoplasmiques qu'on trouve parfois après la coloration de Pappenheim et qu'on considère probablement comme thrombocytes pathologiques, sont identifiés comme parties de paramyéloblastes ou paramyélocytes. La disposition de la chromatine des ombres de Gumprecht en forme de colliers lors de l'étalement des frottis laisse supposer que les chromosomes existent comme individus aussi dans les noyaux de l'interphase, bien qu'ils se présentent dans un état déspiralisé au maximum.

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Fundación Hematológica Mar del Plata, Argentina

Substitutive Inhibitory Therapy in Constitutional Non-Spherocytic Erythrocytic Disease

By Enrique Rewald

Although large tracks of population have not been investigated, there is no doubt about the extensive distribution of hemolytic diseases, specially due to the high incidence of abnormal hemoglobins in Mediterranean countries and in certain areas of Asia and Africa. The severity of the homozygous state induced interesting speculations about the disappearance of some isolated communities (1)

The difference between hypo- and hyper-regenerative anemia is fundamental. With identical low Hb. concentration a patient is able to live normally (simple aplastic disease) or his activities are limited by easy fatigability (hyperactive erythropoiesis) (fig 1)

In adults, bone marrow volume is about 2.600 cc. (2) equal to 5/9 of blood volume. Normally half of it is in activity with 6 / of nucleated cells (3) Fat marrow can be replaced in a few days (4) due to the regenerative potential of hemopoiesis. Crosby and Akeroyd (5) calculated that as much as 6 to 8 times the normal amount of red cells might be produced daily (fig 2) In cases of ineffective erythropoiesis (specially thalassemias) there is a discrepancy between the erythroid hyperplasia and the poor red cell delivery

) Increasing of basophilic erythroblast proliferation rate.

b) Delay of maturation to orthochromatic erythroblast (due to defectuous Hb. synthesis)

Many abortive forms never reach peripheral blood and numerous nucleated and reticulated red cells remain very shortly (hours) in circulation (infant mortality theory), as indicated by early labelled bile pigment (6) and the double exponential nature of Cr^{51}-erythrocyte survival curves (7)

d) By-products during states of erythropoietic acceleration may accentuate the metabolic effort (8, 9)

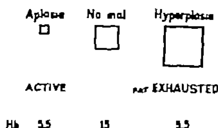


Fig. 1 Comparison of erythropoietic area

Bone marrow is an important center of erythroclasia, due to the size and rich content of phagocytic reticulum cells. This function is largely increased in hemolytic disease, specially when ineffective erythropoiesis produces many frustrated cells.

Fig. 2 Disproportion between erythroblastic cell mass and red cells in circulation. Increase of total marrow volume in hemolytic disease

Associated hyperemia contributes to enlarge the marrow volume and seems to be the main cause of frequent bone pains (10). Increase of blood supply appears both in erythroblastic proliferation and excessive destruction (i. e. sudden enlargement of spleen during hemolytic crisis). In absence of secondary hypersplenism, erythropoietic stimulation is often associated with leukocytosis and even leukemoid reaction. Increase of platelets is not rare. Osteoblastic proliferation is common and may contribute to structure changes and deformities of bones. Nevertheless the main skeletal alterations are due to osteoporosis. Widening of marrow cavities causes thinning (even disappearance) of trabecles and cortex.

Persistent erythroblastic reaction may be suddenly *interrupted* by maturation arrest, apparently provoked by the increased requirements of some nutritional factor. Such aplastic crisis are frequently associated with pancytopenia and secondary infections.

The enormous erythropoietic mass with high mitotic rate reminds of a progressing malignant tumour. In consequence, increased demand of metabolites has to be expected. The high consumption of folic acid predisposes to megaloblastic complications (11). Substraction of nutritive factors seem to be the main cause of chronic fatigue, dystrophy in infancy, impairment of preadolescent acceleration of growth and hypogonadalism. Low uropepsin excretion has been found in COOLEY's disease (12), suggesting hypophyseal insufficiency. Tendency to chronic leg ulcers in adults is also a characteristic feature. The magnitude of continuous compensatory effort is specially reflected on the general condition during active development periods, hyponutrition or chronic toxic infectious events.

In spherocytosis, the sudden normalization of erythropoiesis after splenectomy leads to a striking improvement of these symptoms and the patient feels better than ever. This fact seems to reveal a clear priority of compensatory red blood cell production to the detriment of other tissues. To avoid this damage in other hemolytic diseases, we suggest to obtain arrest of bone marrow reaction by regular transfusions, in order to maintain hemoglobin at a non stimulating level.

a) Inhibition [illegible] erythropoiesis was demonstrated in normal recipients after a blood transfusion equal to 40% of total blood volume (13). This was confirmed also in pernicious anemia (14)

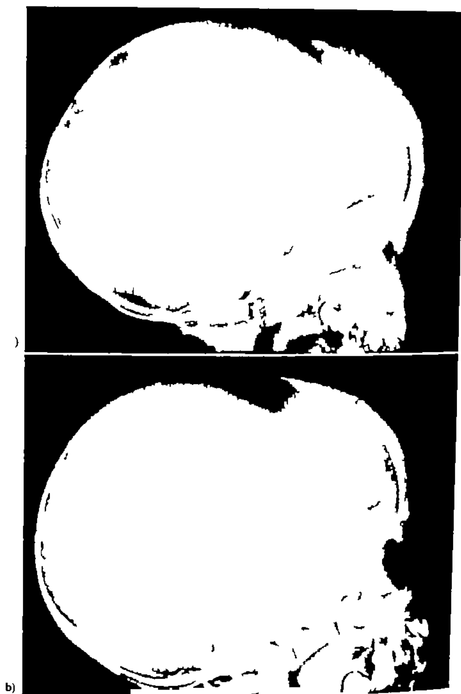


Fig 2. Comparison of skull roentgenograms before ( ) and after (b) 90 day interval. Diminishing of radiating triations and osteoporosis. Improvement of abnormal frontal protruberance.

sickle cell disease (15) and thalassemia (16). In the latter blood requirement to induce erythropoietic rest and disappearance of Hb.F is relatively small, suggesting that erythropoietic effort is avoided by the replacement of abnormal red cells. Perhaps pituitary hypofunction contributes to the diminished need of Hb level. In our patients we observed not only reticulocyte decrease after transfusion, but also normalization of leukocytosis and disappearance of bone pains.

b) Rebound phenomenon. The inhibitory period is relatively short and followed by erythropoietic hyperactivity as indicated by a rise of Hb level in the third week after transfusion (16). We had occasion to observe this phenomenon.

Boy (2) with unequivocal severe Mediterranean anemia, was maintained almost without reticulocytes by transfusions at regular two-week intervals. From the beginning there was striking improvement of splenomegaly and after 3 months clear evidence of head shape normalization. Diminution of prominent frontal and parietal bones as well as other skeletal alterations (fig. 3). Increase of weight.

His return was delayed due to bad weather. Sixteen days after the last transfusion intense bone pains began (specially in skull and pelvis). Forty-eight hours later we found an Hb. level of 11.6 gr%, reticulocytes 0.3% and plasma clear. In contrast there was intense cellularity in bone marrow with evident predominance of erythroblasts, many of them maturated up to orthochromatic stage. A transfusion elevating Hb. to 15 gr% caused two days later the disappearance of bone pains. Equal to erythroblastic maturation period.

In conclusion, bone pains indicated the start of a rebound phenomenon in absence of accentuated anemia. If we want to keep our patients symptom-free it seems to be wise to avoid or interrupt the rebound phenomenon by transfusion.

c) Extracorpuscular hemolytic factor. When blood requirements become excessive, splenectomy as corrective measure must be considered. SMITH et al. (17) demonstrated the return to pre-transfusional status of normal red cell survival after this operation. There is no doubt that new techniques in antibody detection will be helpful for prolonging the treatment in severe hemolytic disease.

d) Siderosis. Repeated transfusions must be associated with elimination of iron excess (i.e. Desferrioxamine B) (18).

e) Other risks such as serum-transmitted hepatitis oblige to select carefully the cases according to the severity of their symptoms.

The concept of compensatory erythropoietic depression helps us to understand that WINTROBE (19) considers maintainance of Hb at nearly normal levels as the only successful treatment for

chronic leg ulcers in hemolytic disease* Clinical remission in thalassemia by regular transfusions have been published by several Italian authors (20) Our results indicate that the influence on bone marrow by this method is equivalent to splenectomy in spherocytosis.

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Summary

A reduction of erythropoiesis in sever hemolytic disease as obtained maintaining hemoglobin constantly bove the stimulation level, in order to avoid) that priority in metabolite consumption by proliferating erythropoietic mass provokes endocrine and trophic disturbances, as well as easy fatigue b) skeletal deformations resulting from bone marrow expansion,) consequences due to destruction of defectuous red cells (i.e. sickling)

Résumé

Les auteurs obtiennent une réduction de l'érythropoïèse chez des cas d'une maladie hémolytique grave en maintenant constamment la concentration de l'hémoglobine au-dessus d seuil de la stimulation. On évite ainsi) que la priorité métabolique du tissu provoque des troubles endocriniens et trophiques ainsi qu'une légère fatigue b) des déformations squelétiques à la suite de l'expansion médullaire et) les suites de la destruction des érythrocytes pathologiques, p. ex. des drépanocytes.

Zusammenfassung

Bei schwerer hämolytischer Krankheit wurde eine Reduktion der Erythropoese erzielt, indem der Hämoglobingehalt konstant über der Reizschwelle gehalten wurde. Damit konnte emieden werden, daß die Priorität des proliferierenden Gewebes im Stoff echsel zu endokrinen und trophischen Störungen, sowie zu leichter Ermüdbarkeit führte Ferner bildeten sich Skelettdeformationen zufolge der Knochenmarksexpansion zurück Ein es erer Z eck ist Folgeerscheinungen des Abbaues defekter Erythrocyten B Sickling) zu hindern

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European Society of Haematology

2nd Session of the Board of the Standardizing Committee

1. The session was held on 10th and 11th February 1964, in Freiburg i. Br. with Prof. Dr L. Heilmeyer in the chair. The meeting was attended by G. Astaldi, Ch. G. de Boroviczény, J. F. Coster, S. M. Lewis, J. Spaander and B. Thorell, members of the Board, and A. H. Holtz, who was invited as an expert consultant.

2. It was noted with satisfaction, that the Transactions of the Committee and Proceedings of Symposium XVIII of the Ninth Congress of the European Society of Haematology in Lisbon has been published as *Bibliotheca Haematologica* Fasc. 18 (S. Karger Basel/New York 1964). Dr Boroviczény was commended on his valuable work as editor. It was decided, to send the full publication to all persons who had attended the Symposium and to the leading medical journals, and to send reprints of the Transactions of the Committee with its recommendations on haemoglobinometry to National, Regional and International liaison officials.

3. There had been correspondence with liaison officials in 28 countries in Europe. The majority had replied. National or regional committees were already established or were being organised in almost all the countries or regions from whom replies had been received.

4. In order to promote national and international contacts and the establishment of national or regional committees in terms of rule 5 of the foundation (see p. 109 of the Transactions) it was decided that an extra-ordinary session of the assembly (as defined in rule 2 of the foundation) should be held in Stockholm on the occasion of the Congress of the International Societies of Haematology and Blood Transfusion. This session will take place presumably on Sunday 30th August at 2 p.m. in Stockholm in the presence of the board, accredited representatives of established national or regional committees, observers from European countries who have not yet established committees and, as observers, a number of invited organisations and individuals (see 5 and 6).

5. Accredited membership of the assembly will be accorded to one delegate from each established national or regional committee which has been approved by the board, provided that the delegate is accepted as representing his national or regional committee. The committees will be asked to nominate their delegates on these terms before 31th July 1964. Each delegate will be invited personally by the board to attend and participate in the assembly with full voting rights. Each delegate will receive a copy of the transactions of the Lisbon meeting (see 2 above) and will be informed of the agenda of the assembly (see 7).

6. The following observers will be invited to attend and participate in the assembly but no voting rights: President and Secretary-General of the European Society of Haematology, World Health Organisation, International Standards Organisation etc., Liaison officials from European countries or regions where a committee has not yet been established, interested specialists from areas outside Europe. These observers will receive a copy of the Lisbon transactions if not already in possession of a copy as per 2.

7. At the assembly accredited representatives of national or regional committees will be requested to report on their committees. All members of the assembly will be invited to participate in discussion on the proposed decision on haemoglobinometry (see also 9) but acceptance of any decision will be by vote of only accredited representa-

tatives or national or regional committees and the board. The representatives will also be asked to make suggestions for future activities of the Standardizing Committee. The board will be available to meet all members of the assembly for discussions.

8. It was decided that the assembly in Stockholm would be an extra-ordinary one. Thereafter assemblies will normally be held biannually within the framework of the Congresses of the European Society of Haematology.

9. Assemblies will be reserved for decisions on proposals for standardization. Scientific discussion in advance of such decisions will take place in symposia during congresses. These symposia will be an integral part of the congress, and in accordance with the rules of the congress. Suggestions for subject matter for the symposium will be made by the board to the Organizing *Committee of the Congress*. Dr Boroviczény reported, that he will be the moderator of symposium on *general principles of standardization, documentation and normal values in haematology*' during the Stockholm Congress of the International Society of Haematology. It was also decided to try to arrange, within this symposium, for scientific discussion on the haemoglobin molecule by group of *invited specialists, and to postpone any further decisions by the assembly with regards to* of the recommendation concerning haemoglobinometry (see p. 110 of the transactions) until this problem has been clarified at the scientific level.

10. Rijks Instituut voor de Volksgezondheid in Utrecht, was asked to prepare stock of 50 l of hemiglobincyanide solution for use as reference standard. The solution should be equivalent to approximately 37 µmol or 60 mg% haemoglobin, dispensed in 10 ml ampoules and controlled yearly by five laboratories through the agency of the members of the board.

11. Rijks Instituut voor de Volksgezondheid was invited to organize new trial of haematological procedures and to report the results at the symposium during the Stockholm Congress (see 9). It was decided to enlarge the trial to include the participation of laboratories outside Europe.

12. Distribution *of this report*. *Members of the Board*, *President of the European Society of Haematology*. Secretary-General of European Society of Haematology. Contact addresses of National or Regional Standardization Committees, accredited Members of the Assembly in Stockholm. Medical journals: Acta haemat., Blood, Blut, Brit. J. Haemat., Brit. med. J. Lancet, Minerva med., Scand. Journals, Vox Sanguinis etc.

Libri

J. C. Reisder **Groupes Sanguins et Immunisation Groupale au Congo.** Editions Arscia, Bruxelles/Librairie Maloine Paris 1963. 239 p., 66 tab.

Die vorliegende Monographie gibt einen umfassenden Überblick über die Verteilung der ABO-Blutgruppen und des Rhesusfaktors D bzw. D^u bei den verschiedenen Volksstämmen des belgischen Kongos. Es handelt sich um einen wesentlichen Beitrag zur Blutgruppenanthropologie der schwarzen Rasse.

A. Hässig, Bern

Vernon M. Ingram. **The Hemoglobins in Genetics and Evolution.** Columbia University Press, New York/London 1963. 165 p., many figs., Price 45 s.

Dr. Ingram's book is an excellent survey of the central role which hemoglobin research has played in recent years in molecular biology and evolution. Physicians who have not had a chance to keep up with the breathtaking advances in this field will find this book an admirable introduction to some of the newer concepts. Using data from synthetic studies and X-ray diffraction, a clear outline of the synthesis and three dimensional structure of the molecule is given. The abnormal hemoglobins are described with special attention to the fingerprinting technique of peptide analysis. The most interesting sections of the book deal with the author's ideas of hemoglobin evolution and the switch from fetal to adult hemoglobin.

The close homology in chemical make-up of the various types of normal hemoglobin chains makes it likely that they have arisen from a common ancestral molecule. Comparative study of the amino acid sequence of animal hemoglobins has shown marked similarities between species. It is now becoming possible to trace evolutionary developments and affinities by analysis of proteins or peptides. This technique promises to be a most powerful one for better understanding of evolution in many other areas as already demonstrated for some pituitary hormones.

The fetal-adult hemoglobin switch is an excellent model for embryologic differentiation. All cells of the body have the same chromosomal and genic make-up. Yet some genes act during development while others are "turned off". Usually the gene product can only be analyzed with difficulty. In the hemoglobin system, fairly generous amounts of protein are available. Considering both thalassemia and the "high HbF" syndrome, the author speculates on the possible role of control mechanisms in hemoglobin synthesis. His ideas are most stimulating and will be of great interest to hematologists interested in thalassemia. A chapter is devoted to Hb A_2, the normal minor hemoglobin synthesis.

This reviewer found intense intellectual pleasure in reading Ingram's book. The mark of the outstanding scientist is his ability to generate experimentally testable hypotheses of fundamental problems. Dr. Ingram, whose fame rests on his experimental work, brilliantly displays his capacity to penetrate to the core of problems in this area and is willing to stick his neck out in his interpretations. The book is highly recommended for every physician interested in the biologic aspects of medicine.

A. G. Motulsky, Seattle, Washington

Acta haemat. 31 313–324 (1964)

From the Second Medical Department, University of Helsinki and the Finnish Red Cross Blood Transfusion Service Helsinki

Gastric Lesion in Iron Deficiency Anaemia

By E. Ikkala and M. Siurala

In iron deficiency anaemia there is a rather high incidence of gastritis, which is presumably responsible for the common occurence of achlorhydria in this condition (1 4 5 6 8 10 13) There is, however considerable disagreement as to the significance of gastritis in iron deficiency anaemia. Moreover the number of patients followed up after adequate iron treatment has been rather small.

The purpose of the present investigation was to study the functional and morphological state of the gastric mucosa in iron deficiency anaemia and, in particular the response of the gastric mucosa to adequate iron treatment

Methods

The following haematological examinations were performed

Peripheral blood picture consisting of haemoglobin and microhaematocrit determinations, erythrocyte, reticulocyte leukocyte and platelet counts, and differential count of the leukocytes

Bone marrow examination including sideroblast stain The latter as modified from the method of Kaplan et al (9) by staining the smears with May-Grünwald-Giemsa before fixation and Prussian blue staining.

Serum iron 14) and total iron binding capacity (18)

Iron absorption test Serum iron was determined two and four hours after administration of 600 mg (four tablets) of ferrous sulphate.

The following gastro-intestinal examinations were performed

Gastric biopsy using the bechlaff suction biopsy tube (Richard Wolf, West Germany) Three biopsies were taken from each patient. Thus, 300 specimens were obtained from 100 patients. All but 4 specimens were representativ and well preserved The specimens were fixed in 5 neutral formaline and stained with haematoxylin an Gieson and haematoxylin eosin

The acidity of the gastric contents was determined with Topfers reagent in samples taken 30, 60 and 90 minutes after administration of 7 5 mg of Hamalog[R] (Lilly) per 10 kg of body weight

Uropepsin determination 12 hours over-night excretion was determined (19)
d-Xylose tolerance test: urinary excretion during five hours following load of 25 g of d-Xylose.

Patients

One hundred consecutive patients treated at the First and the Second Medical Clinics, University of Helsinki, who suffered from definite iron deficiency anaemia, were included in the series. The patients were selected on the following criteria

82 patients had haemoglobin values below 12 g/100 ml, serum iron below 40 γ/100 ml, and total iron binding capacity (TIBC) above 350 γ/100 ml 17 patients had TIBC values of 288–348 γ/100 ml but met the remaining criteria and responded adequately to iron treatment one patient who had taken iron tablets before admission had serum level of 54 γ/100 ml but fullfilled the criteria in other respects.

Of the 17 patients who had TIBC values of 288–348 γ/100 ml, sideroblasts were counted in 12. The count was below 5 in these patients.

Patients who had been operated upon for some gastroduodenal disease were not included in the series. None of the patients suffered from active gastroduodenal ulcer or gastric tumours.

There were 6 males and 94 females. The mean age was 33.3 and the range 15–0 years.

The etiology of iron deficiency was considered obvious 12 patients Intestinal malabsorption (6) melaena of unknown origin (2) ulcerative colitis (?), anorexia ner osa (1) and oesophageal stenosis (1). Fourty-two patients had had profuse menstrual nasal or haemorrhoidal bleedings, which probably were of some etiologic significance Most of these 42 patients and 13 additional patients had lived on an apparently iron deficient diet. There remained 31 patients in whom nothing of etiologic importance could be established. Stool examination for occult blood the time of the present study ga negative results in all but the two patients with ulcerative colitis and the two patients with melaena of unknown origin.

Sixteen patients suffered from diseases which might have had some deleterious effect upon the gastric mucosa. Of these 5 had thyrotoxicosis, 8 cardiovascular diseases, and 3 gallbladder disease All but two of the patients had gastritis. In addition, one patient with atrophic gastritis suffered from rheumatoid arthritis

Results

Haematological findings The bone marrow was examined in 91 patients. In most cases there was slight to moderate hypercellularity with an increase in nucleated red cells, in 18 patients the finding was regarded as normal and the finding in 1 patient is described in the case report on page 320 The sideroblasts were counted in samples from 73 patients. The incidence of sideroblasts in haemoglobin-containing normoblasts was in 11–15 in 3,5 – 10 % in ?% and below 5 in 50 samples.

The individual values of haemoglobin, mean corpusc haemoglobin concentration serum iron and total iron b capacity are listed in figures 1 4 The leukocyte count was 4 000 mm³ in ?7 4 000–8,000 mm³ in 65 and above 80

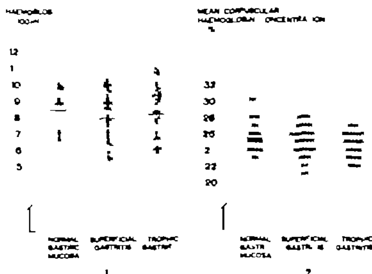


Fig. 1 Distribution of the haemoglobin values by the state of the gastric mucosa.
Fig. 2 Distribution of the mean corpuscular haemoglobin concentrations by the state of the gastric mucosa.

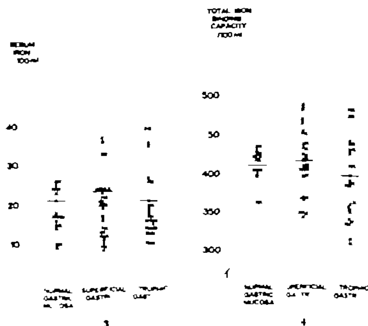


Fig. 3 Distribution of the serum iron values by the state of the gastric mucosa.
Fig. 4 Distribution of the total iron capacity values by the state of the gastric mucosa.



Fig 5. Gastric biopsy of patient S. V., female aged 52. Severe inflammatory signs and moderate loss of normal body glands is visible (Van Gieson, × 70)

Fig 6. Gastric biopsy of the same patient as in fig 5 six months after iron treatment. The body glands appear normal. There is, however still inflammatory cell infiltration beneath the surface epithelium (Van Gieson, 70)

in 8 patients. The platelet count was low (100 000–150 000/mm³) in 3 normal 151 000–450 000/mm³) in 78, and slightly or moderately increased 451 000–800 000/mm³) in 18 patients. One patient showed pronounced thrombocytosis.

Morphological state of the gastric mucosa. Bioptical studies revealed gastritis in 75 of the 100 patients studied. The distribution of the patients according to the state of the gastric mucosa was as follows normal gastric mucosa 25 patients superficial gastritis inflammatory cells below the surface with or without infiltration of the deeper layers) (fig 6) 39 patients atrophic gastritis (loss of normal body glands) (fig 5 7 8, 9) 36 patients slight atrophic gastritis 21 patients moderate atrophic gastritis 12 patients severe atrophic gastritis (complete or almost complete loss of body glands) fig 9) 3 patients.

Epithelial changes were in general associated with marked inflammatory signs The prevailing form of inflammatory cells was the lymphocyte They often occurred in aggregates with a germinative centre A number of such lymphomas were found in 23 pa-



7 8 9

Fig. 7. Gastric biopsy of patient K. A., female, aged 51. There is moderate loss of normal body glands (Van Gieson, × 70)

Fig. 8. Gastric biopsy of the same patient as in fig. 7 six months after iron treatment. There is marked loss of normal body glands with intestinal and pseudopyloric metaplasia (Van Gieson, 70)

Fig. 9. Gastric biopsy of patient E. N., female, aged 16. The normal body glands are replaced by intestinal tubules (Van Gieson, 70)

tients, mainly in those with atrophic gastritis. In addition to lymphocytes, plasma cells, eosinophils and non-eosinophilic granulocytes were commonly seen. Very heavy eosinophilia was present in 4 and a somewhat less severe one in 25 cases. An increase of eosinophilia was noted with increasing severity of gastritis. The occurrence of large numbers of inflammatory cells in general, and of granulocytes in particular gave the impression of an "active" process in two-thirds of the patients with gastritis. Thus, in patients with atrophic gastritis the inflammatory cell infiltration was severe in 15, moderate in 9 and slight or almost absent in 12 patients.

Atrophic gastritis was commonly associated with various kinds of metaplasia. Thus, intestinal metaplasia (intestinal type of glands, fig. 9) was noted in 13, and pseudopyloric metaplasia (pyloric type of glands, fig. 8) in 30 cases.

There was no clear correlation between the occurrence and the degree of gastritis, on the one hand, and the age of the patients, on the other hand. The mean age of patients with a normal gastric mucosa was 35.7 ± 11.6 (SD) and that of patients with gastritis 35.2 years (superficial gastritis 28.4 ± 11.6 and atrophic gastritis 42.5 ± 14.9 years). The age difference between the normal and the atrophic gastritis groups was statistically insignificant. The incidence of gastritis in men was the same as in women, however, the number of men was too small to permit any conclusions.

The morphological changes in the gastric mucosa are correlated with the haematological findings in figs. 1–4. It appears that there is no correlation whatever between the state of the gastric mucosa, on the one hand, and the haemoglobin, MCHC, serum iron and TIBC values, on the other hand. Neither was any correlation encountered between the quality and quantity of inflammatory cell infiltration and the haemotological values. The duration of iron deficiency, as judged from previous iron treatments, seemed not to bear any relation to the occurrence of gastritis.

The occurrence of angular stomatitis, papillary atrophy of the tongue and koilonychia in various conditions of the gastric mucosa is shown in table I. It appears that changes in the tongue and nails

Table I

Number of patients with angular stomatitis, papillary atrophy of the tongue and koilonychia, distributed by the state of gastric mucosa

	State of gastric mucosa		
	Normal	Superficial gastritis	Atrophic gastritis
Total number of patients	25	39	36
Angular stomatitis	7	7	12
Papillary atrophy of the tongue	1	1	8
Koilonychia	1	2	8

are more often encountered in patients with atrophic gastritis than in other mucosal conditions.

Functional state of the gastric mucosa. The Histalog test performed with 98 patients revealed achlorhydria in 28 patients, peak secretion of hydrochlorid acid of 1–50 mEq/l in 24 patients, peak secretion of 51–100 mEq/l in 44 patients, and more than 100 mEq/l in 2 patients. As shown in fig. 10, there is a distinct correlation between the peak secretion values and the morphological changes in the gastric mucosa.

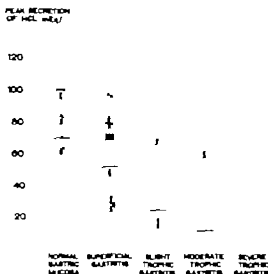


Fig. 10. Distribution of the peak secretion of HCl alone by the state of the gastric mucosa.

Uropepsin excretion was examined in 78 patients and revealed values below 10 UP units/h in 33 patients and values above 10 UP units/h in 45 patients. Again a clear correlation was obtained with the morphological findings. The results of Histalog tests and uropepsin determinations showed no correlation either with the haematological findings or with the duration of the iron deficiency

d Xylose and iron absorption tests d Xylose excretion was examined in 76 patients. Low values (below 5g/5h) were found in 13 patients. Three of these had other signs of malabsorption, two had cardiovascular diseases and one thyrotoxicosis.

The iron absorption test was performed with 98 patients. In only two patients the serum iron level remained below 50g/100ml after the iron load. In 14 patients it increased to 50–150 γ/100 ml, in 26 patients to 151 250 γ/100 ml and in the remaining 56 patients to more than 250 γ/100 ml These results showed no correlation with the state of the gastric mucosa.

Follow-up examinations Sixty patients were re-examined 3–11 months (mean 6 months) after the first examination Iron deficiency anaemia was considered to be cured in 30 patients they all had haemoglobin above 12 g/100 ml, serum iron above 50 γ/100 ml,

and TIBC below 350 γ/100 ml Improvement of iron deficiency anaemia was established in 18 patients they fulfilled the above criteria either for haemoglobin or for serum iron and TIBC. Eleven patients who had discontinued the treatment were still anaemic at the time of re-examination. In one patient the iron treatment led to manifestation of polycythemia vera. The case history of this patient is presented below

A 58-years old widow who for many years had been occasionally treated with iron preparations, was hospitalized for anaemia. On admission her blood picture was as follow Haemoglobin 6.2 g/100 ml, erythrocytes 3.36 mill./mm PCV 27 leukocytes 5,900 mm with a normal differential count, and platelets 1,000,000–1,400,000 mm³ Serum iron was 17 /100 ml and TIBC 360 /100 ml. The bone marrow was highly hypercellular with an increase in nucleated red cells, including many immature forms. The megakaryocytes were very numerous.

Thus, in addition to a typical finding of iron deficiency anaemia, the thrombocytosis and the marrow hypercellularity with numerous megakaryocytes suggested some myeloproliferative disease. Peroral iron treatment of three months duration brought about a definitely polycythaemic blood picture: Haemoglobin 15 g/100 ml, erythrocytes 6.82 mill./mm PCV 57 leukocytes 6,200 mm³ with slight shift to left in the differential count, and platelets 1,060,000 mm³

Table II

Changes in gastric mucosa compared with haematological changes in 47 patients re-biopsied after iron treatment.

Haematological finding	Histological changes: Some improvement	Unchanged	Some worsening
Anaemia cured	4	16	5
Anaemia improved	2	10	3
Still anaemic		4	3

Gastric biopsy was performed in 47 patients at the re-examination The results are given in table II Some improvement was noted in 6 and some worsening of the gastritis in 11 patients. It also appears that improvement was established only in patients in whom the iron deficiency anaemia was improved or cured. However the changes in any direction were far from evident Thus, complete restitution of a diseased gastric mucosa or development of severe atrophy was not noted in any case Hence it seems that the morphological state of the gastric mucosa remained essentially unchanged in spite of others use successful treatment When the response of the different mucosal elements to iron treatment was examined, it appeared that no marked epithelial changes had occurred. On the other hand the number of inflammatory cells the more labile

elements—varied somewhat at different examinations. These changes, however bore no relationship to the haematological findings.

Table III

Changes in the peak secretion of HCl in Histalog test compared with the haematological changes in 26 patients re-examined after iron treatment.

Haematological finding	Changes in the peak secretion of HCl		
	Increased	Unchanged	Decreased
Anaemia cured	3	12	1
Anaemia improved	2	5	
Still anaemic		3	

The Histalog test was performed with 26 patients (table III) Free hydrochloric acid was demonstrated at the re-examination in 3 patients with achlorhydria. In additional 2 patients, hydrochloric acid secretion increased from 3 to 57 and from 18 to 96 mEq/l respectively None of the 5 patients had anaemia at the time of the re-examination. On the other hand, corresponding histological improvement did not occur in them

Uropepsin excretion was re investigated in 22 patients. There was an increase in 3 and a decrease in 2 patients.

Discussion

Gastritis was found in 75 of 100 patients with iron deficiency anaemia. This finding is in accordance with previous reports (1 4 5 6, 8, 10, 13) The incidence of gastritis is known to increase with age and to be somewhat more common in females than in males. Most of the present series were females and many were over 50 years of age. Hence it seems reasonable to assume that in some individual cases the coexistence of anaemia and gastritis was merely incidental. However the high incidence of gastritis and the lack of age correlation suggest that in most cases there existed some causal relationship between the two conditions. This conclusion, reached also by other authors, is not invalidated by the observed lack of correlation between the haematological and bioptical findings. It should be noted that the comparison was based upon the results of one examination only The duration of gastritis could not be determined and the estimation of the duration of iron deficiency rested on the case history data. Provided that the two conditions are causally interrelated, the estimation of these two parameters —duration of iron deficiency and duration of gastritis—would be of

the greatest importance. However it is not possible to obtain reliable data on the duration of these diseases.

There is no general agreement as to the question whether gastritis predisposes to or is caused by the iron deficiency. Lees and Rosenthal (10) Bothwell and Finch (3) and Wood and Taft (22) are of the opinion that the gastric lesion precedes the anaemia. Wood and Taft suggest that gastritis leads to iron deficiency by decreasing appetite and by bleeding. The old concept that gastritis leads to achlorhydria and by this means interferes with iron absorption is supported by the recent studies of Williams (20) and Goldberg et al. (7). On the other hand, according to Moore and Dubach (11), Pirzio-Biroli et al. (12) and Biggs et al. (2) achlorhydria seems to have no effect on the absorption of iron salts and dietary iron. Thus the role of achlorhydria in the etiology of iron deficiency cannot be definetely stated. The lack of response of gastritis to adequate treatment of the iron deficiency might speak for the primacy of the gastric lesion. However the follow up studies of Siurala et al. (15) and Siurala and Vuorinen (17) suggest that gastritis, once established, tends to remain and to progress, and that a complete healing is rare. Accordingly the lack of gastric response to treatment excludes by no means the possibility that gastritis is caused by iron deficiency as has been suggested by Witts (21), Badenoch et al. (1) and Davidson and Markson (6). Iron deficiency is known to cause angular stomatitis and changes in the nails and tongue. In agreement with Badenoch (1) there was a good correlation between these changes and the gastric mucosal lesion in the present study also. In addition some of the present patients showed signs of disturbed function of the small intestine (d-Xylose test). Hence it is possible that gastritis is only a part of the general response of the whole oro-oesofageo-gastro-intestinal mucosa to iron deficiency. It may be mentioned, moreover, that Siurala and Tawast (16) found inflammatory and/or regressive mucosal changes in the large intestine of most patients with iron deficiency anaemia.

It appears that no uniform explantation can be given for the coexistence of gastritis and iron deficiency. This is only natural, since iron deficiency is by no means the sole cause of gastritis and vice versa. Both conditions may have different etiological factors, some of which however they may have in common. Many patients with iron deficiency anaemia are living under conditions such as

dietary deficiency dietary indiscretions and psychic stress, which may predispose to iron deficiency on the one hand, and to gastritis, on the other hand Gastritis in turn by causing loss of appetite, abdominal distress, bleeding and achlorhydria, may aggravate the iron deficiency This, again, may further impair the gastric mucosal condition. Thus, under the influence of several factors a vicious circle may develop between gastritis and iron deficiency

Acknowledgement. This study was supported by grant from Sigrid Jusélius Foundation.

Summary

Seventy-five of 100 patients with iron deficiency anaemia were found to have gastritis, which was superficial in 39, slight atrophic in 21 moderate atrophic in 12 and severe atrophic in 3 patients. The occurrence of gastritis correlated neither with the age of patients nor with any of the haematological parameters used. With advancing degree of gastritis there was distinct decrease in HCl secretion and uropepsin excretion. Achlorhydria was present in 28 of 98 patients. Koilonychia and papillary atrophy of the tongue occurred mainly in patients with atrophic gastritis. D-Xylose absorption was impaired in 13 of 76 patients.

A second gastric biopsy was performed in 47 patients after approximately 6 months iron treatment. Some morphological improvement was noted in 6 and some worsening in 11 patients. However signs of improvement or aggravation of the mucosal state were rather slight, and in general the state of the gastric mucosa remained essentially unchanged. In one patient iron treatment led to manifestation of polycythaemia vera.

Résumé

Chez 75 de 100 malades souffrant d'une anémie ferriprive une gastrite peut être trouvée. Chez 39 elle est superficielle, faiblement atrophique chez 21 moyennement atrophique chez 12 et gravement atrophique chez 3 malades. Il n'y a pas de relations entre l'existence d'une gastrite et l'âge des malades ou les données hématologiques. Parallèlement à la gravité de la gastrite on peut trouver une diminution notable de la sécrétion d'acide chlorhydrique et de l'excrétion de l'uropepsine. 28 de 98 malades présentent une achlorhydrie. La koilonychie et l'atrophie papillaire se trouvent surtout chez les malades atteints d'une gastrite atrophique. La résorption de la d-Xylose est modifiée chez 13 de 76 malades.

Une seconde biopsie de la muqueuse gastrique est pratiquée chez 47 malades après un traitement par le fer pendant 6 mois. Six présentent une amélioration alors que onze présentent une aggravation. En général les signes d'une amélioration ou aggravation de l'état de la muqueuse gastrique ne sont que discrets et l'état de la muqueuse reste en général inchangé. La thérapie par le fer provoque la manifestation d'une polycythémie chez un des malades.

Zusammenfassung

Bei 75 von 100 Patienten mit einer Eisenmangelanämie wurde eine Gastritis festgestellt. Sie war oberflächlich bei 39, leicht atrophisch bei 21, mäßig atrophisch bei 12 und schwer atrophisch bei 3 Patienten. Das Vorkommen der Gastritis stand weder zum Alter der Patienten noch zu einem der hämatologischen Befunde in Beziehung. Mit zunehmender Schwere der Gastritis fand sich eine deutliche Verminderung der HCl-Sekretion und der Uropepsinausscheidung. Eine Achlorhydrie fand sich bei 28 von 98 Patienten. Koilonychie und Papillenatrophie der Zunge kamen vor allem bei Patienten mit atrophischer Gastritis vor. Die Resorption von d-Xylose war bei 13 von 76 Patienten gestört.

Bei 47 Patienten wurde nach einer Eisentherapie von 6 Monaten eine zweite Magenbiopsie vorgenommen. Eine Besserung fand sich bei 6 und eine Verschlimmerung bei 11 Patienten. Jedoch waren die Zeichen der Besserung oder Verschlimmerung im Zustand der Magenschleimhaut nur geringgradig und im allgemeinen blieb der Befund im wesentlichen unverändert. Bei einem Patienten führte die Eisentherapie zur Manifestation einer Polycythaemia vera.

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Unterschiedliche saure Phosphatasereaktion von Monozyten und Exsudatmakrophagen

Von H. Braunsteiner und F. Dienstl

Die Beziehung zwischen Monozyten und Makrophagen im Gewebe und in Ergüssen ist vielfach diskutiert worden. Nach Ansicht zahlreicher Untersucher können Makrophagen aus Monozyten entstehen, zum Teil wird sogar die Identität beider Zellarten vertreten. Diese Beziehung ist auch für das noch ungelöste Problem der Monozytenentstehung von Bedeutung, wo sich 3 Hypothesen, die Entstehung aus myeloischen Zellen, die Existenz eines eigenständigen monozytären Systems und schließlich die Existenz eines mono-histiozytären Systems, das auch die Makrophagen umfaßt, gegenüberstehen (1).

Rein morphologische Argumente können kaum die endgültige Abklärung bringen, da sowohl Monozyten als auch Makrophagen im Gewebe und Exsudaten eine erhebliche Fähigkeit zu gestaltlichen Abwandlungen besitzen. Auch durch funktionelle Kriterien, worunter in erster Linie die Phagozytosefähigkeit zu verstehen ist, konnte bisher kein sicherer Unterschied gefunden werden.

In den letzten Jahren sind zytochemische Untersuchungen in zunehmendem Maße zur Differenzierung einzelner Zellinien verwendet worden. So zeigen beispielsweise nur Granulozyten eine ausgeprägte Naphthol-AS-D-Chlorazetatreaktion, die mit einer Proteaseaktivität dieser Zellen in Beziehung stehen dürfte. Monozyten und retikulohistiozytäre Zellen sowie Exsudatmakrophagen weisen eine stark ausgeprägte, mit α-Naphthol-, Naphthol AS- und AS-D-Azetat nachweisbare unspezifische Esterasereaktion auf (5).

In vorhergehenden Untersuchungen war es bereits aufgefallen, daß retikulohistiozytäre Zellen im Knochenmark und in der Milz eine stark positive saure Phosphatasereaktion mit Naphthol AS-BI

phosphat als Substrat geben, während Monozyten des peripheren Blutes nur schwach positiv sind (4). Diese Untersuchungen wurden nunmehr auf Exsudatmakrophagen erweitert.

Methodik

Meerschweinchen im Gewicht von 300–400 g wurden i.p. mit 10–20 ml einer 0,01 %-Glykogenlösung injiziert. 24 Stunden bzw. 4 und 5 Tage nach der Injektion wurde die Bauchhöhle mit physiologischer Kochsalzlösung gespült, die Spülflüssigkeit bei 900 Touren/Min. abzentrifugiert, die sedimentierten Zellen in 4 % Albuminlösung aufgeschwemmt und auf Objektträger ausgestrichen. Bei 3 Patienten mit Pleuraexsudat wurden in gleicher Weise die Zellen sedimentiert und unmittelbar ausgestrichen. Die Ausstriche wurden nach May-Grünwald-Giemsa, zur Darstellung der unspezifischen Esterase mit Naphthol AS-D Acetat, wodurch auch eine eindeutige Identifizierung gegeben ist, und zur Darstellung der sauren Phosphatase nach der folgenden Anweisung (3) gefärbt.

Luftgetrocknete Ausstriche werden 30 Sekunden in kaltem 60% Aceton fixiert, gespült und in folgender Lösung, die frisch zubereitet werden muß, 3 Stunden inkubiert. Zu 5 ml Michaelis-Veronalacetat-Puffer pH 9,71 werden 1 ml in Dimethylformamid gelöstes Naphthol-AS-BI phosphat und 13 ml Aqua dest. hinzugegeben. Dann werden 1,6 ml Hexazonium-p-Rosanilin-Lösung zugefügt und am pH-Meter ein pH-Wert von 5,0 eingestellt. Nach der Inkubation wird wieder gespült, anschließend 8 Minuten in Mayers Hämalaunlösung gestellt, wieder gespült und in Glyceringelatine eingebettet.



Abb. 1 Makrophagen aus dem Peritonealexsudat vom Meerschweinchen. Stark positive saure Phosphatasereaktion. Das Zytoplasma ist leuchtend rot.

Ergebnisse

Alle Makrophagen sowohl in den Peritonealexsudaten beim Meerschweinchen als auch in menschlichen Pleuraexsudaten wiesen eine außerordentliche starke saure Phosphatasereaktion auf (Abb. 1). Sie entspricht in ihrer Intensität der von speichernden Retikulumzellen im Knochenmark. In Zellen des strömenden Blutes, insbesondere in Monozyten konnten wir niemals eine Reaktion von ähnlicher Intensität beobachten.

Diskussion

Aus diesen Untersuchungen geht hervor, daß zwischen Monozyten im Blut und Exsudatmakrophagen zytochemisch ein deutlicher Unterschied hinsichtlich der sauren Phosphatasetätigkeit unter Verwendung von Naphthol-AS-BI-phosphat als Substrat besteht. Monozyten geben eine schwach positive, während Makrophagen aus Pleura- und Peritonealexsudatflüssigkeit von Mensch und Meerschweinchen eine sehr stark positive Reaktion geben und sich somit wie retikulo-histiozytäre Zellen im Gewebe verhalten.

Die saure Phosphatase findet sich zusammen mit einer Reihe von weiteren sauren hydrolytischen Enzymen in den sogenannten Lysosomen. Darunter versteht man eine polymorphe Gruppe elektronenmikroskopisch nachweisbarer Cytoplasmapartikel, die von einer einfachen Membran umgeben sind. So lange die strukturelle Integrität dieser Partikel gewahrt ist, zeigen die sauren Hydrolasen keine wesentliche Aktivität gegen von außen herangebrachte Substrate, erst nach der Zerstörung der Membran kommt es zur vollen Fermentaktivität. Phagozytierende Zellen sind besonders reich an Lysosomen, die sich nach der Phagozytose in die Umgebung des phagozytierenden Objektes ansammeln und ihren Inhalt in die Phagozytosevakuole entleeren. Durch diesen Mechanismus bleibt die Zelle vor der Selbstverdauung geschützt. Wahrscheinlich werden diese Hydrolasen jedoch auch extrazellulär sezerniert und bewirken derart die Auflösung von geschädigtem Gewebe bzw. beim Gewebsabbau freiwerdender Substanzen.

Es ist nicht zulässig, aus diesem zytochemisch unterschiedlichen Verhalten auf einen prinzipiellen Unterschied zwischen Monozyten und Exsudatmakrophagen bzw. retikulohistiozytären Zellen zu schließen. Die physiologische und pathophysiologische Bedeutung der Leukozytenphosphatasen ist bisher weitgehend ungeklärt.

Es ist möglich daß unter bestimmten Bedingungen eine funktionelle Adaptation der Makrophagen erfolgt und die zytochemisch nachweisbare saure Phosphataseaktivität stark ansteigt. Eine solche Steigerung wurde während der Phagozytosetätigkeit beschrieben (5) In unseren Untersuchungen war zwar keine wesentliche Makrophagozytose feststellbar doch läßt sich etwa eine gesteigerte Pinozytose morphologisch kaum nachweisen. Eine Aktivitätszunahme der sauren Phosphatase in Zellkulturen von Monozyten wurde kürzlich beschrieben (Fischer und Gropp)

Zusammenfassung

Exsudatmakrophagen von Mensch und Meerschweinchen weisen unter Verwendung von Naphthol AS-BI-phosphat als Substrat eine viel stärkere saure Phosphataseaktivität als Blutmonozyten auf. Sie verhalten sich wie retikulohistiozytäre Zellen im Gewebe

Summary

Macrophages from human and guinea-pig exudates show a much more pronounced acid phosphatase reaction than blood monocytes when naphthol AS-BI phosphate is used as substrate. They thus react similarly to reticulohistiocytes in tissue

Résumé

L activité de la phosphatase acide des macrophages d'un exsudat de l'homme ou du cobaye est plus forte que celle des monocytes sanguins lorsqu' on utilise le naphthol AS-BI phosphate comme substrat. Les réactions des macrophages ressemblent à celles des cellules réticulo-histiocytaires du tissu.

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Effect of Hypoxic Guinea Pig Plasma Upon Erythropoiesis in the Polycythemic Mouse and Polycythemic Guinea Pig*

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A variable response of the guinea pig to erythropoietin has been reported by different authors. OSMOND et al. (1) obtained no erythropoietic response, but did obtain a granulocytic response following administration of active human urine extract to the normal guinea pig. GARCIA AND SCHOOLEY (2) however demonstrated an erythropoietic response in the hypertransfused guinea pig to an active urine extract obtained from the same patient and recently we have demonstrated a response of the hypertransfused guinea pig to active human urine extract (3). Others (4, 5, 6) have reported both response and failure of response of the guinea pig to active extracts.

Because of these contradictory results we have determined the response of the sensitive polycythemic mouse to pooled plasmas from guinea pigs made hypoxic for variable periods and have compared this response with that obtained when the same pooled plasmas were injected into hypertransfused polycythemic guinea pigs.

Materials and Methods

1. *Assay in polycythemic mice of plasmas from guinea pigs exposed to hypoxia.* One group of male Swiss mice, weighing 25–30 gm were kept in an atmosphere of 10 % O_2 for 3 weeks (7). On removal their mean hematocrit was 72.1 %. Five days later when erythropoiesis was suppressed with an Fe^{59} RBC incorporation of less than 0.5 % and an absence of peripheral reticulocytes, one ml of separately pooled plasma from guinea pigs exposed to 24, 48 or 72 hours of hypoxia was given subcutaneously to three groups of polycythemic mice (average dose 4 ml/100 gm body weight). Phenol extracts of the same batches of pooled plasma were given to additional groups of these polycythemic

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mice. Sixty hours later 0.5 $\mu c Fe^{59}$ Cl_3 was injected into the tail vein. One half ml of blood was obtained by cardiac puncture 72 hours later and counted in scintillation counter

A second group of mice were made polycythemic by intraperitoneal transfusions on two consecutive days of one ml of an 80 suspension of washed homologous erythrocytes suspended in isotonic saline. By the sixth day following the second transfusion erythropoiesis was suppressed, (erage hematocrit 62) and one ml of pooled hypoxic guinea plasma (4 ml/100 gm body weight) was injected subcutaneously. The Fe^{59} RBC incorporation was subsequently determined by the same procedure described above. Duplicate microhematocrits were btained on all mice.

2. *Assay in the hypertransfused guinea pig of plasmas from guinea pigs exposed to hypoxia.* Polycythemia was induced in inbred guinea pigs averaging 275 gm in eight by transfusing them intraperitoneally on tw consecutive day with an 80 suspension of homologous washed erythrocytes suspended i isotonic saline. On the fifth day following the last transfusion when reticulocytes had disappeared from the blood and the animals ere strikingly polycythemic, 4 ml/100 gm body weight of pooled plasma obtained from normal guinea-pigs and guinea pigs made hypoxic for arying periods of time were injected intraperitoneally. Preliminary experiments had shown that doses lower than 4 ml/100 gm did not produce response. Forty-eight hours later the injected animals and uninjected controls recei ed 1 μc Fe^{59} Cl_3 in 0.1 ml saline by cardiac puncture. The guinea-pigs were exsanguinated 48 hours later and 1 ml aliquot of blood was counted in scintillation counter. Duplicate microhematocrits were obtained. Autopsy showed no evidence of extra asation of blood into the pericardium or mediastinum.

3. *Exposure of guinea pig to hypoxia and collection of plasmas.* Guinea pigs were placed in an air-sealed tank containing 10 O and 90 N_2. Groups of animals were then removed at tervals of 6, 10, 14 48 and 72 hours and immediately bled by cardiac puncture. The blood was centrifuged and the plasmas obtained from each time interval group were pooled and frozen at —20 C until use. Normal guinea pig plasma as collected in the same manner.

Phenol extraction of plasma was carried out according to the method of Low (8) Extracts were concentrated by flash ev poration to 25 of the original olume of plasma and injected subcutaneously into the hypoxic-induced polycythemic mice as described abov

Results

1. *Response of hypertransfused polycythemic guinea pigs to plasmas from guinea pigs exposed to hypoxia as measured by Fe^{59} RBC incorporation.* The Fe^{59} RBC incorporation was essentially the same in animals receiving normal guinea pig plasma as it was in the uninjected animals (table I fig 1) Plasma from guinea pigs exposed to hypoxia for 24 hours, when injected into hypertransfused polycythemic guinea pigs caused an enhancement of 528 in Fe^{59} RBC incorporation as compared with the effect of normal plasma from guinea pigs not exposed to hypoxia. Plasma from guinea pigs exposed to hypoxia for 48 hours, although showing increased activity (97 $p < 0.05$) over the control level was considerably less active than that from guinea pigs exposed to hypoxia for 24 hours. Plasma from normal guinea pigs and from animals exposed to hypoxia for

hours did not produce a significantly different incorporation of Fe^{59} into erythrocytes from that of the uninjected control. A comparison with Fe^{59} red cell incorporation in *normal* guinea pigs (table I) shows that erythropoiesis in the hypertransfused guinea pig did not return to normal levels following injection of any of these plasmas from hypoxia-exposed guinea pigs.

Table I

Response of the hypertransfused guinea pig to plasma obtained from guinea pigs exposed to 24 48 and 72 hours of hypoxia

Test materials injected	No. of animals	Mean weight (gms)	HCT %	% Fe^{59} Incorporation	% Increase Fe^{59} incorporation of control
Uninjected	4	290.5	53.3 ± .32	3.43 ± 0.998	
Normal Guinea Pig Plasma	3	281.3	54.3 ± 2.6	3.68 ± 0.55	0
24-hr hypoxic plasma	3	280	53.7 ± 1.2	18.12 ± 2 41 (P < .05)	528
48-hr hypoxic plasma	3	272	55 7 ± 2.3	6.66 ± 0.44 (P < .05)	97
72-hr hypoxic plasma	3	278	57 7 ± 3.8	6.13 ± 2.72 (P < 1)	89
Normal Uninjected Guinea Pig	2	281	40.5 ± .5	52.3 ± 5.26	

48-hour Fe^{59} RBC Incorporation assay

All values are them mean ± standard error (S. E.).

** Hematocrits were obtained on the day of assay

2. *Response of hypoxia-induced polycythemic mice to plasmas from guinea pigs exposed to hypoxia.* As can be seen in tables II and III and figs. 1 and 2 maximal Fe^{59} incorporation into the erythrocytes of these polycythemic mice, occurred following injection of plasma from guinea pigs exposed to hypoxia for 24 hours. The magnitude of this response was markedly elevated above the control level and was considerably greater in the hypoxia induced polycythemic mouse than in the hypertransfused polycythemic guinea pig. Throughout these experiments the amount of plasma injected was 4.0 ml/100 gm of the recipient animal. It should be emphasized that Fe^{59} RBC incorporation was determined 48 hours after Fe^{59} injection in the guinea pigs due to early escape from polycythemic suppression (3) and 72 hours after Fe^{59} injection in the mice when maximum response is obtained. Some activity was also present in th plasma from guinea pigs exposed to hypoxia for 48 hours In th hypoxia induced polycythemic mouse this greatly exceeded the

control value and was statistically significant ($p < 0.05$) The plasma from guinea pigs exposed to hypoxia for 72 hours showed, statistically minimal erythropoietic activity ($p < 1 > 05$)

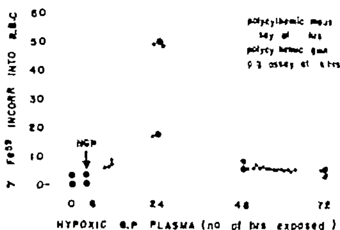


Fig 1 Comparison of the incorporation of Fe^{59} into erythrocytes of the hypertransfused guinea pig (●) and of the hypoxia-induced polycythemic mouse (○) following injection of plasma from guinea pigs exposed to 24 48 and 72 hours of hypoxia. Assay performed 48 hours after injection of Fe^{59} Cl_3 into the guinea pig and 72 hours after injection of Fe^{59} Cl_3 into the polycythemic mouse.
NGP Normal guinea pig plasma control.

Table II
Hypoxia-induced polycythemic mouse assay Response to plasmas obtained from Guinea pigs exposed to 24 48 and 72 hours of hypoxia

Guinea pig plasma injected (hours of hypoxia)	No. of animals	HCT %	% Fe59 incorporation
Uninjected	3	59 ± 2.6	0 64 ± 77
24	4	59 ± 2.6	3 08 ± 15 (P < .05)
48	4	54 ± 2	8.63 ± 1.93 (P < .05)
72	4	53.5 ± .91	4 26 ± 1 43 (P < 10)

72-hour Fe^{59} RBC incorporation.
All values are expressed as the mean ± standard error (S. E.)

In comparison to the above phenol extraction of guinea pig plasmas caused a loss of erythropoietic activity (table III) the extract from guinea pigs exposed to hypoxia for 24 hours was the only one retaining a significant minimal amount of activity

Table III

Hypoxia-induced polycythemic mouse assay. Response to phenol extracted guinea pig plasmas

Plasma injected (Phenol extracted) hours of hypoxia	No. of animals	HCT %	% Fe^{59} incorporation
24	4	53.2 ± 2.39	2.43 ± 0.56 (P<.05)
48	2	54.5 ± 0.5	1.59 ± 0.4 (P>.1)
72	4	55.5 ± .5	1.42 ± .42 (P>.1)
Uninjected	3	59 ± 2.6	0.64 ± .077

72-hour Fe^{59} RBC incorporation.
Values are expressed as the mean ± standard error (S. E.).

Table IV

Transfusion-induced polycythemic mouse assay. Response to plasmas obtained from guinea pigs exposed to 6, 10, 24, 48 and 72 hours of hypoxia

Materials Injected	No. of animals	HCT %	% Fe^{59} RBC incorporation
Uninjected	7	57 ± 1.48	0.39 ± .042
Normal Guinea Pig Plasma	4	59 ± 0.67	1.04 ± .61
Hypoxic Plasma			
6 hours	4	60 ± 1.98	1.43 ± .2 (P>.1)
10 hours	4	63.0 ± 2.1	3.95 ± 2.04 (P>.1)
24 hours	3	59 ± 1.61	30.72 ± 4 (P<.05)
48 hours	4	54 ± 2.45	2.10 ± 1.85 (P>.1)
72 hours	5	52 ± 1.48	0.66 ± .1 (P>.1)

72-hour RBC Fe^{59} incorporation.
Values are expressed as the mean ± standard error (S. E.)

3. *Comparison of the transfusion induced polycythemic mouse assay and the hypoxia-induced polycythemic mouse assay in testing plasmas from hypoxic guinea pigs.* Fig. 2 and tables II and IV demonstrate the greater sensitivity of the hypoxia induced polycythemic mouse assay in measuring erythropoietic activity in pooled plasmas from guinea pigs exposed to 24, 48 and 72 hours of hypoxia. The small titres of activity in the latter two plasmas were demonstrable only when the hypoxia-induced polycythemic mouse assay was used. Incorporation of Fe^{59} into erythrocytes following administration of plasma from animals exposed to 6 hours of hypoxia was not

significantly different than that of hypertransfused mice who received normal guinea pig plasma. There appeared to be some increased incorporation of Fe^{59} into erythrocytes of hypertransfused mice following administration of plasma from animals exposed to ten hours of hypoxia but the standard error was large. The shape of curves of both assay animals were essentially the same. It is evident that the polycythemic mouse will respond to erythropoietically active plasmas from hypoxic guinea pigs.

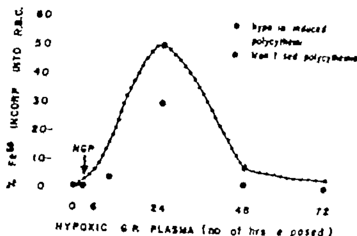


Fig. Comparison of the transfusion induced polycythemic mouse assay (●) with the hypoxia-induced polycythemic mouse assay (O) following injection of plasma from guinea pigs exposed to hypoxia. Assay was performed 72 hours after the injection of $Fe^{59}Cl_3$. NGP Normal guinea pig plasma control.

72-hour Fe^{59} RBC incorporation.

Discussion

The polycythemic mouse assay, whether prepared by transfusion of homologous red cells given intraperitoneally or by induction of polycythemia through chronic exposure to hypoxia, has been shown to be extremely sensitive to erythropoietically active substances (7, 9–12) in biological fluids. Whether the guinea pig will respond to other species' erythropoietin has been controversial (1, 6). The effect of guinea pig erythropoietin upon erythropoiesis in other species has not been reported. An increase in the number of erythroblasts was observed in hanging drop bone marrow cultures from guinea pigs or rats on addition of serum or of boiled filtrates of plasma from anemic patients and guinea pigs (16). It has been shown that plasma from animals exposed to hypoxia is erythro-

poietically active. STOHLMANN (17) demonstrated an erythropoietic response in the starved rat assay to plasma from rats made hypoxic. This erythropoietic activity of hypoxic plasma has been confirmed by others (13–15 18, 19) and the maximal activity in plasma was found after 12–24 hours of hypoxia.

The data presented here show hypoxic guinea pig plasma to be erythropoietically active in the polycythemic mouse and to a lesser extent in the hypertransfused polycythemic guinea pig The peak activity is found in plasma from guinea pigs made hypoxic for 24 hours. The same dose of 4 ml/100 gm body weight was employed both in the mouse and guinea pig assay A comparison of the quantitative response of the mouse and guinea pig cannot be completely evaluated since a 48-hour Fe^{59} RBC incorporation assay was used in the guinea pig due to the early escape of this assay animal (3) from erythropoietic suppression produced by polycythemia and a 72 hour Fe^{59} RBC incorporation assay was used in the mouse, at which time the maximum response occurs following $Fe^{59}Cl_3$ administration. Recently we have also shown human urine extract from a hypoplastic anemia patient to be active in the transfused guinea pig (3)

As shown in fig 2, the chronic hypoxia induced polycythemic mouse assay was more sensitive than the transfusion induced polycythemic mouse assay especially to smaller titers of erythropoietic activity present in plasmas obtained from guinea pigs exposed to 48 and 72 hours of hypoxia.

Whether there is a molecular difference of the erythropoietin of guinea pigs and of other species has not been ascertained. Previous difficulties in obtaining an erythropoietic response to active human urine extracts in the guinea pig may be partly explained by a species specific difference of molecular structure. However the lack of sensitivity of the previously used assay animals to erythropoietically active materials may have also played a role. *It has also been suggested that human erythropoietin may be more* antigenic when administered to the guinea pig causing impairment of full erythropoietic response (2)

Conclusions

1 Plasma from guinea pigs exposed to hypoxia enhanced incorporation of Fe^{59} into erythrocytes of hypertransfused polycythemic mice and guinea pigs and hypoxia-induced polycythemic mice.

2 Maximal erythropoietic activity in plasma developed after guinea pigs were exposed to hypoxia for 24 hours although some activity was also present in plasma from guinea pigs exposed to 48 and 72 hours of hypoxia.

3 Phenol extraction of erythropoietically active guinea pig plasma caused a marked loss of this activity

4 The hypoxia induced polycythemic mouse assay proved more sensitive to erythropoietic activity of guinea pig plasma than the hypertransfused polycythemic mouse assay

Summary

The erythropoietic response of the polycythemic mouse to plasma obtained from guinea pigs exposed to hypoxia was compared with that of the hypertransfused polycythemic guinea pig. Plasma obtained from guinea pigs made hypoxic for 24 hours induced the greatest response of Fe^{59} incorporation into the red cells in both assay animals. The sensitivity of the assay for erythropoietin was compared in the hypoxic induced polycythemic mouse and in the hypertransfused polycythemic mouse The hypoxic induced polycythemic mouse assay proved more sensitive detecting smaller titers of erythropoietin activity in the plasmas from guinea pigs subjected to hypoxia for 48 and 72 hours. Most but not all of the erythropoietic activity was lost in phenol extracts of plasma from guinea pigs rendered hypoxic for 24 hours.

Résumé

Les auteurs comparent la réaction de l'érythropoïèse des souris polycythémiques à l effet du plasma de cobayes exposés à une hypoxie à celle de souris après la pré-transfusion L'incorporation du Fe^{59} aux erythrocytes est influencée le plus fortement par le plasma de cobayes exposés pendant 24 heures à une hypoxie. Puis on compare la sensibilité de la détermination de l'érythropoïétine chez les souris polycythémiques après hypoxie et par hypertransfusion. Le test est plus sensible chez les souris après hypoxie parce qu il permet de déterminer des titres plus faibles de l'activité de l'érythropoïétine dans le plasma des cobayes exposés à l'hypoxie pendant 48–72 heures. Presque toute l'activité de l'érythropoïétine est perdue par l'extraction au phénol chez les cobayes exposés pendant 24 heures à l'hypoxie

Zusammenfassung

Die Reaktion der Erythropoese polycythämischer Mäuse auf Plasma von Meerschweinchen die einer Hypoxie ausgesetzt worden waren, wurde verglichen mit derjenigen übertransfundierter Meerschweinchen. Der Einbau von Fe^{59} in Erythrocyten wurde bei beiden Versuchstieren am stärksten beeinflusst durch Plasma von Meerschweinchen, die während 24 Stunden einer Hypoxie ausgesetzt worden waren. Bei der durch Hypoxie und bei der durch Übertransfusion polycythämisch gemachten Maus wurde die Empfindlichkeit der Erythropoietinbestimmung verglichen. Dabei erwies sich der Test bei der ersteren als empfindlicher indem er geringere Titer der Erythropoietinaktivität im Plasma von Meerschweinchen nachweisen ließ, die ... während

48–72 Stunden einer Hypoxie ausgesetzt worden waren. Nahezu die gesamte Erythropoietinaktivität ging in Phenolextrakten von Plasma derjenigen Meerschweinchen verloren, die während 24 Stunden einer Hypoxie ausgesetzt worden waren.

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Aus der Neurochirurgischen Universitätsklinik Zürich (Prof. Dr. H. Krayenbühl)

Über die intrakranielle Ausbreitung der Leukämien unter besonderer Berücksichtigung der leukämischen Meningopathie*

Von A. Brielmann

Blutkrankheiten führen nicht selten zu eindrücklichen neurologischen Syndromen und zu schweren pathologisch-anatomischen Veränderungen im zentralen und peripheren Nervensystem. Besonders die malignen Erkrankungen des lymphatischen und myeloischen Systems nehmen hier einen besonderen Platz ein. Williams et al. (44) haben dies in einer Monographie eindrücklich darstellen können. Der Ablauf der Leukämien hat sich nun aber seit Einführung der Chemotherapie mit Corticosteroiden und Antimetaboliten nicht unwesentlich verändert, indem, besonders bei den akuten Formen im Kindesalter, neue Verlaufsarten aufgetreten sind. Wir haben den Fall eines fünfjährigen Knaben mit Stammzellenleukämie beobachtet, der unter der neuen Chemotherapie einen solch typisch andersartigen Verlauf nahm und unter den Zeichen des chronischen Hirndruckes gestorben ist. Da auch in der Literatur in letzter Zeit ähnliche Fälle publiziert wurden (8, 9, 16, 28, 29, 30, 31, 35, 36, 39, 40, 44, 46, 47), scheint uns eine kasuistische Mitteilung lohnenswert.

Anamnese

Der 6jährige Knabe M. R. erkrankt im Frühjahr 1955 [1] an einer Stammzellenleukämie, wahrscheinlich einer akuten lymphatischen Leukämie. Es wird eine Behandlung mit Bluttransfusionen, Aminopterin und Meticorten begonnen. Dabei kommt es zu einer Remission der Erkrankung bis Januar 1956, als der [illegible] Schub [illegible]. Nach Unterbrechung der Behandlung mit den gleichen Medikamenten [illegible] Kopfschmerzen und Erbrechen ein. Diese Symptome werden erst als Ausdruck einer Commotio nach leichtem Kopftrauma gedeutet. Bei der Lumbalpunktion findet man allerdings eine deutliche Pleocytose und stellt nun die Diagnose [illegible]. Deutliche Erniedrigung des Liquorzuckers. Nachdem sich Stauungspapillen und epileptische Anfälle nachweisen lassen, wird der Patient der Neurochirurgischen Universitätsklinik [illegible]

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* Herrn Prof. [illegible] zu seinem 70. Geburtstag gewidmet.

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tätsklinik Zürich (Prof. H. KRAYENBÜHL) überwiesen. Der Knabe zeigt neurologisch neben der doppelseitigen Amaurose die typischen Zeichen des chronischen Hirndruckes. Er wird unter der Diagnose einer Liquorabflußstörung im Bereiche der Brücke ventrikulographiert, wobei ein symmetrischer Hydrocephalus internus inkl. des 3. Ventrikels gefunden wird. Bei der anschließenden cerebellaren Exploration läßt sich aber nur ein eigentümlich weißliches Gewebe in den etwas verdickt scheinenden *Meningen* der hinteren Schädelgrube nachweisen. Eine genaue histologische Diagnose kann aus der Probeexcision nicht gestellt werden. Der postoperative Verlauf ist kompliziert durch das Auftreten eines epiduralen Haematoms. Völlige Remission der Leukämie im peripheren Blut und im Knochenmark. Der Exitus erfolgt plötzlich, unerwartet, am 42. Tage nach Spitaleintritt an Atemlähmung. Die Sektion (Patholog. anat. Institut der Universität Zürich, Prof. E. UEHLINGER, S. N. 1350/56) ergibt das überraschende Resultat einer schweren diffusen leukämischen Infiltration des ganzen Subarachnoidalraumes bis zur Cauda equina, Infiltrate in der Hypophyse und den inneren Organen, keine Metaplasien in der Gehirnsubstanz (Abb. 1 2)



Abb. 1 Paraleukoblastäre Infiltrate bzw. Metaplasien in der basalen Arachnoidea (S.N. 1350/56, 6j. Knabe) Frontalschnitt durch die Großhirnhälften auf Höhe der Hirnschenkel. Pannusartige Verdickung der Arachnoidea im Bereiche der Hirnbasis, besonders deutlich sichtbar in der Fossa interpeduncularis. Hydrocephalus internus.

Diskussion

Bei den Leukämien kommt es zu den verschiedensten Veränderungen im zentralen und peripheren Nervensystem. VIRCHOW selbst (zit. 28) beschrieb als einer der Ersten perivasculäre Infiltrate in der Mark und Rindensubstanz, sowie in subependymalen Bezirken und in der Leptomeninx (ältere Literatur 12, 19 5 34) SCHWAB (37) stellte bis 1935 146 Fälle der Weltliteratur zusammen. Darunter finden sich auch die 9 Fälle von TRÖMNER UND WOHLWILL (41) wel



[illegible] Paraleukoblasten-Infiltrate in der basalen Arachnoidea [illegible] Auslegung entlang den Gefäßen ohne eigentliche Infiltrationen bzw. Metaplasien in der Hirnsubstanz. Schnitt durch die Pons. [illegible]

che vor allem dadurch interessant waren, daß die oft sehr deutlichen, zentral nervösen Veränderungen, bedingt durch die Leukämie fast keine neurologischen Ausfallserscheinungen hervorgerufen hatten. (Weitere ausführliche Zusammenstellungen 8, 13 18, 25) Aus allen diesen Zusammenstellungen geht hervor daß die Leukosen weitaus am häufigsten von allen Blutkrankheiten Veränderungen am zentralen Nervensystem verursachen. Sowohl petechiale Blutungen als auch große Massenblutungen stehen bei weitem an erster Stelle und führen auch fast immer den Tod in wenigen Tagen herbei. Die Entstehungsweise und die Art dieser Blutungen sind in neuester Zeit eingehend untersucht worden (10, 11 27) Wir werden später bei der Besprechung der Pathogenese der uns vor allem interessierenden Meningopathia leucaemica nochmals darauf zurückkommen. Im Hinblick auf die Blutungen ist vor allem auch noch an die epi und subduralen Hämatome zu denken, besonders dann wenn man vor der Durchführung intracranieller Eingriffe steht. Sie bilden dabei eine nicht zu vergessende Gefahr wie dies unser eigener Fall eindrücklich demonstriert. Weiterhin finden sich Infiltrate und Veränderungen an den Gehirnnerven und peripheren Nerven (1 2 5 22, 38, 41) oft mit Riesenzellbildungen (42) schließlich seltenerweise auch tumorartige Wucherungen (21 23 26) Nach Ansicht der meisten Autoren zeigen sich leukämische Infiltrate in der Gehirnsubstanz nur selten ohne Zusammenhang mit den Gefäßen wenn sie vorhanden sind, liegen sie vor allem in der weißen und seltener in der grauen Substanz. Bei den tumorartigen Bildern handelt es sich meistens um extradural gelegene Geschwulstknoten, welche, ähnlich der Hodgkin schen Erkrankung zu Kompressionen meist des Rückenmarkes führen

Die von den Leukämien im zentralen Nervensystem erzeugten klinisch neurologischen Symptome sind ebenso mannigfach wie die pathologisch-anatomischen Veränderungen. Es finden sich geordnet nach der Häufigkeit, folgende Symptome Halbseitenlähmungen und epileptische Anfälle meist bei Blutungen, radikuläre Reizerscheinungen und polyneuritische Krankheitsbilder bei Infiltraten der Spinalganglien und der peripheren Nerven (4 22) Herpes zoster bei Infiltraten der Spinalganglien, schwere symptomatische Trigeminusneuralgien bei Umwachsung und Infiltration des Ganglion semilunare (eigener Fall, nicht publiziert) psychische Veränderungen teilweise bedingt durch die Infiltrate und die fast alle Leukämien begleitende Anämie klassische Hirndruckzeichen

mit Stauungspapillen, Kopfschmerzen und Erbrechen bei den vorwiegend im Subarachnoidalraum gelegenen und zu Liquorabflußstörungen führenden Infiltraten, Retinaveränderungen (ausführliche Literatur 34). Gelegentlich werden auch Hinterstrangdegenerationen im Sinne der funikulären Myelose als seltene Symptome zitiert. HEILMEYER (18) glaubt, daß diese Hinterstrangdegenerationen als «Aufbrauchspernicosa» zu deuten seien. ROUQUES (34) hingegen lehnt echte Hinterstrangdegenerationen bei Leukämien ab, ebenso ERBSLOH (11), der behauptet, daß der Goll- und Burdach'sche Strang lediglich durch Bildung kleinster myelomalacischer Herde degenerieren.

Die Häufigkeit dieser neurologischen Symptome wird nun von den vielen Autoren mit großen Unterschieden in den Statistiken angegeben. SCHWAB UND WEISS (37) fanden sie bei 22,5 %, LEIDLER UND RUSSELL (25) bei 35 %, DIAMOND (8), LIESE (26) sogar bei 50 %, BRANDT (5) wiederum nur bei 8,6 % ihrer Fälle. Leidler behauptet sogar, daß man bei ca. 80 % von an Leukämien verstorbenen Patienten pathologisch anatomische Veränderungen im Gehirn finden könne. Das Eindrücklichste an all diesen Zahlen ist schließlich nur die Tatsache, daß viel mehr zentralnervöse pathologisch anatomische Veränderungen als klinisch-neurologische Symptome gefunden werden. Die Uneinheitlichkeit all dieser Zahlen beweist, daß das untersuchte Material zu wenig einheitlich ist, und daß man das Zentralnervensystem bei Leukämien systematischer untersuchen und diese Befunde mit dem klinisch neurologischen Bild vergleichen sollte. Was die einzelnen Leukämiearten betrifft, so besteht praktisch kein Unterschied zwischen akuten und chronischen, sowie lymphatischen und myeloischen Leukämien (24, 25, 37, 39, 43, 44). Man erhält aber doch den Eindruck, daß die intracraniellen resp. die zentralnervösen Veränderungen etwas häufiger bei akuten als bei chronischen und hier wieder mehr bei lymphatischen als bei myeloischen Leukämien vorkommen. Die Blutungen treten häufiger bei myeloischen bzw. bei Stammzellenleukämien, also bei akuten Verlaufsformen auf, begreiflicherweise deshalb auch häufiger im Kindesalter bzw. im jugendlichen Alter als in vorgerückten Jahren. LASZLO (24) behauptet, daß sämtliche neurologische Komplikationen bei jüngeren Leuten häufiger seien, und daß vor allem auch die Männer dabei mehr betroffen seien als die Frauen. Eine diesbezügliche Bestätigung konnten wir in der Literatur nicht finden.

Es ist nun noch interessant, etwas mehr auf die Pathogenese resp. die Histogenese der leukämischen Infiltrate und Gefäßveränderungen im Zentralnervensystem einzugehen. Handelt es sich dabei überhaupt um Infiltrate, oder sind alle diese Zellenansammlungen speziell diejenigen im Subarachnoidalraum als eigentliche Metaplasien zu deuten? Im Sinne von APITZ (3) handelt es sich bei den Leukämien um eine Systemkrankheit, um eine Ansiedlung neoplastischer und metaplastischer Zellen in ihrem potentiellen Lebensraum. Die Ansiedlung leukämischer Zellen im Gehirn erfolgt nun vorwiegend in einem mesenchymalen Gewebe, das reich an Histiozyten und adventitiellen Zellen ist, also Zellformen, welche, wie die Reticulumzellen, von multipotenten Zellen abstammen. Auch das Gewebe der Plexus chorioidei setzt sich aus den gleichen mesenchymalen Elementen wie das die Hirngefäße begleitende Bindegewebe zusammen. In den ersten vier Monaten des Embryonallebens findet zum Beispiel in den Plexus chorioidei noch eine reichliche Haematopoese statt, wie das KAPPERS (7) mit sehr schönen Abbildungen hat beweisen können. *Es darf nun wohl mit* Wahrscheinlichkeit angenommen werden, daß dieses mesenchymale Gewebe auch in postembryonalen Zeiten immer noch multipotente Stammzellen enthält, welche eventuell auf einen leukämischen Stimulus (40) gleich reagieren wie die Zellen im Knochenmark. Dieses gleiche Gewebe stellt ja auch die Matrix der nicht so seltenen sarcoiden Wucherungen des reticulo-histiozytären Systems im Gehirn dar (adventitielles, embryonales Mesenchymalnetz, 6, 15, 33). Auf Grund dieser Ausführungen glauben wir entgegen den Annahmen von ERBSLÖH (11), daß es sich bei den mannigfachen leukämischen Zellansammlungen im Gehirn, bzw. im zentralen Nervensystem nicht um hämatogen oder lymphogen entstandene Infiltrate, sondern um eigentlich an Ort und Stelle entstandene Metaplasien handelt. LUDWIG (27) hat mit seinen histologischen Untersuchungen ebenfalls zeigen können, daß sich die Leukämiezellen praktisch nur herdweise in diesem Gefäßbindegewebe finden lassen. Dieses aktive Mesenchym (33) funktioniert im Embryonalleben als blutbildendes Gewebe, postnatal aber übernimmt es die Funktion eines potentiellen Entzündungsgewebes. Bei einer Systemerkrankung wie den Leukämien *oder* den Reticuloendotheliomatosen, Retothelsarkomen usw.) kann es wiederum zur Hämatopoese entarten resp. zur leukämischen oder tumorösen Meta- oder Neoplasie.

Die Verlaufsformen als «Pseudomeningitis leucaemica» oder besser als leukämische Meningopathie beanspruchen nun noch besonderes Interesse. Unser Fall gehört in diese Gruppe und wir haben ähnliche Fälle in der Literatur finden können (vgl. Einleitung). SHAW et al. (39) fanden bei 16,7 % ihrer Fälle mit akuten Leukosen eine «Meningeal Leukemia». Sämtliche Autoren sind sich im Grunde genommen einig darüber, daß die neuen Behandlungsverfahren mit den Corticosteroiden und den Antimetaboliten diese Verlaufsformen der Leukämien verursachen. Es sind allerdings schon vor der chemotherapeutischen Ära Fälle von ausgedehnten leukämischen Infiltraten in den weichen Hirnhäuten bekannt geworden. SULLIVAN (40) stellte 9 solche Fälle der englischen Literatur zusammen. All diesen Fällen gemeinsam ist klinisch das *Auftreten der intracraniellen Drucksteigerung mit Kopfschmerzen*, Erbrechen, Stauungspapillen, cerebellären Symptomen ohne eigentliche hirnlokale Syndrome. Fast ausnahmslos handelt es sich um Kinder in den ersten zehn Lebensjahren. Gelegentlich zeigen sie cushingoide Bilder, auch wenn keine Corticosteroide gegeben worden sind. Als Folge des Hirndruckes finden sich Sprengung der Schädelnähte und Schädelschappern. Daß es sich dabei manchmal nur um eine scheinbare Schädelnahtsprengung handelt, konnten HITZIG UND SIEBENMANN (20) an einem Fall zeigen, bei welchem die Sprengung der Nähte durch leukämische Infiltrate verursacht worden war. In hämatologischer Hinsicht ist bei den meisten dieser *Fälle von leukämischer Meningopathie auffallend, daß sich die* Leukämie sowohl im peripheren Blut als auch im Knochenmark nicht mehr nachweisen läßt, daß also eine scheinbar völlige Remission eingetreten ist. Auch unser Fall zeigt dieses Verhalten. Im Liquor lassen sich neben meist erhöhtem Druck und Gesamteiweißvermehrung gelegentlich neoplastische Blutzellen nachweisen. Ein weiteres Charakteristikum bei der Liquoruntersuchung scheint die Verminderung des Zuckers zu sein. Der Zuckergehalt ist allerdings auch bei sonstigen, andersartigen neoplastischen (carcinomatösen) Veränderungen im Subarachnoidalraum herabgesetzt (eigene Erfahrungen [40]). Der Liquorzuckererniedrigung kommt deshalb bei diesen Fällen *eine beträchtliche diagnostische* Bedeutung zu.

Wie kommt es zu diesen merkwürdigen neuartigen leukämischen Verlaufsformen? Eine definitive Antwort kann nicht gegeben und es können nur Vermutungen geäußert werden. Man darf wohl annehmen, daß dabei Veränderungen an der *Blut-Liquor*

schranke eine große Rolle spielen. Vielleicht werden die Membranen der Blut/Liquorschranke durch die neuen Chemotherapeutica wie 6-Mercaptopurin und die Corticosteroide irgendwie so verändert, daß es auch jenseits dieser Schranke zur metaplastischen leukämischen Entartung kommt. PENTSCHEW (32) glaubt, daß es vor allem dann zu einer Veränderung der Schrankenfunktion komme, wenn das Ionenmilieu diesseits oder jenseits der Schrankenmembran eine entscheidende Änderung erfahre. Es sind allerdings bisher keine wesentlichen, mit den heutigen Methoden meßbare Ionenverschiebungen im Blute bei den untersuchten Fällen bekannt geworden. Auf Grund der pathologisch-anatomischen Untersuchungen von LUDWIG (27) kann man mit Wahrscheinlichkeit annehmen, daß die Gehirngefäßwände bei den Leukämien eine schwere Umwandlung erfahren. Die ganze Gefäßwand erscheint oft wie aufgelockert und aufgelöst. Man könnte daraus folgern, daß eine vermehrte *Durchlässigkeit, sowohl für Stoffe als auch für Zellen* bestünde. Wäre diese etwas mechanistische Auffassung gerechtfertigt, so müßten aber gerade auch die neueren Chemotherapeutica die Schranke etwas besser passieren und die leukämischen Metaplasien günstiger beeinflussen können.

Es ist nun aber auch nach den Untersuchungen von HAMILTON et al. (17) sowie WOLLNER et al. (45) erwiesen, daß nur kleine Mengen von Cytostatica, resp. von 6-Mercaptopurin (Purinethol) und Amethopterin (Methotrexate) in den Liquor gelangen. Auch SULLIVAN (40) gelangte bezüglich des 6-Mercaptopurins zu ähnlichen Ergebnissen: maximal ein Zehntel dieses Stoffes soll die Liquorschranke passieren.

Im Hinblick auf diese letzteren Ausführungen läßt sich auch bezüglich der Therapie kurz das Wesentlichste sagen. Die Cytostatica müssen intrathecal verabreicht werden, wenn man aus ihrer Anwendung bei der intracraniellen Ausbreitung der Leukämien Nutzen ziehen will. Dies wurde schon von einigen Autoren durchgeführt (39, 47). Die gleichzeitige konsequente Anwendung von Steroiden darf aber dabei nicht unterlassen werden (14). Auch die Röntgenganzbestrahlung des Gehirns wird trotzdem nicht zu umgehen sein (39, 40). Es werden im allgemeinen 500–1 000 r/Einzeldosis gegeben, wobei allerdings die häufige intraspinale Ausbreitung der Leukämien nicht miterfaßt wird, was die Rückfalltendenz natürlich beträchtlich erhöht. Eine Mitbestrahlung des Rückenmarkes kommt aber wohl wegen der Gefahr einer Knochenmark

schädigung kaum in Frage. Die drei Behandlungsarten scheinen nach Ansicht der genannten amerikanischen Autoren mit ihren größeren Patientenzahlen ungefähr gleich wirksam zu sein.

Anmerkung: Herrn Prof. H. L[illegible], Medizinische Universitätsklinik Basel, möchte ich für seine wertvollen Ratschläge bei der Abfassung dieser Arbeit herzlich danken.

Zusammenfassung

Beschreibung eines Falles einer akuten Stammzellenleukämie bei einem 5jährigen Knaben, bei welchem unter Therapie mit Aminopterin und Corticosteroiden die Leukämie im peripheren Blut und im Knochenmark in Remission kommt. Unter zunehmenden Hirndruckzeichen stirbt das Kind nach operativer Revision der hinteren Schädelgrube. Die Sektion zeigt eine massive leukämische Aussaat im Subarachnoidalraum bis in die Cauda equina. Es wird kurz auf die intrakranielle Ausbreitung der Leukämien [illegible]gangen unter besonderer Berücksichtigung der Pathogenese der leukämischen Veränderungen. Die speziellen Verlaufsformen der akuten Leukämien im Kindesalter unter der neuen Chemotherapie mit Antimetaboliten und Corticosteroiden werden diskutiert. Die Ursache der merkwürdigen Veränderungen könnte in Ionenverschiebungen diesseits und jenseits der Blut-Liquor- und Blut-Gehirnschranke bestehen. Die einzige einigermaßen wirksame Therapie der leukämischen Meningopathie besteht in der intrathecalen Applikation von Cytostatica, der Verabreichung von hohen Dosen von Steroiden sowie der Röntgenganzbestrahlung des Gehirns.

Summary

A case is described of an acute stem-cell leukemia in a five-year-old boy in whom a remission in the leukemic findings in peripheral blood and bone marrow was induced under treatment with aminopterin and corticosteroids. The child died with signs of increasing intracranial pressure following operative treatment of the posterior cranial fossa. Autopsy showed a massive leukemic metastasis in the subarachnoid space extending to the cauda equina. Brief reference to the intracranial extension of leukemia is made with particular consideration of the pathogenesis of leukemic changes. The particular forms of the course taken by juvenile acute leukemia under the new chemotherapy with antimetabolites and corticosteroids are discussed. The cause of the remarkable alterations may lie in ion shifts on one or the other side of the blood-CSF and blood-brain barriers. The only therapy of leukemic meningopathy which is at all successful is the intrathecal administration of cytostatic drugs, high dosage steroids and irradiation of the brain.

Résumé

Description d'un cas d'une leucémie aigue à cellules-souches chez un garçon de 5 ans, chez lequel une rémission de la leucémie peut être observée dans le sang périphérique et la moelle osseuse pendant le traitement à l'aminoptérine et [illegible] corticostéroïdes. L'enfant mourut avec les signes d'une pression cérébrale progressive après [illegible] chirurgicale de l'étage [illegible] postérieure. A l'autopsie l'on trouva une dissémination leucémique massive dans l'espace subarachnoïdien jusqu'à la hauteur de la cauda equina. L'auteur discute brièvement la dissémination intracranienne des leucémies, en considérant précisément la pathogenèse des modifications leucémiques. Puis l'auteur discute les formes spéciales de l'évolution des leucémies aigues de l'enfant sous l'influence de la chimiothérapie moderne par les antimétabolites et les [illegible]

La cause des modifications remarquables est trouvée dans les migrations ioniques de part et d'autre de la barrière sang/liquide céphalo-rachidien et sang/tissu cérébral. La seule thérapie plus ou moins effective d une méningopathie leucémique est l'application de fortes doses de stéroides et l'irradiation totale du cerveau.

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Haemophagocytic Reticulosis Diagnosed During Life

By S. Varadi, R. R. Gordon and D. Abbott

In 1952 Farquhar and Claireaux (1) observed a rapidly fatal disease *in two siblings undiagnosed during life which they termed* familial haemophagocytic reticulosis. A further sibling was reported in 1958 (2) and a second family in 1963 (4). The affected infants became ill at the age of 8—10 weeks, anorexia, vomiting, irritability, pallor and fever being the presenting features with splenomegaly and hepatomegaly and sometimes lymph node enlargement. The blood showed progressive anaemia, granulocytopenia and thrombocytopenia along with abnormal mononuclears and numbers of smear cells. The histological features were those of a proliferation of phagocytic histiocytes in various organs.

We now report a further case differing only in age and absence of affected sibs, but otherwise showing both clinical and histological findings of familial haemophagocytic reticulosis. As the disease has never before been diagnosed or even suspected in a first affected child or diagnosed by haematological methods in any of the affected children during life, we thought this further case worth recording. The diagnosis was based on the bone-marrow smears which showed numbers of histiocytes phagocytosing erythrocytes, nucleated red cells and, relatively fewer, white cells.

Case Report

A three years old child was admitted with two weeks history of malaise and anorexia with the more recent onset of drowsiness and vomiting. On the day of admission he had developed a profuse crop of spots all over his body. He had previously been perfectly healthy as were his parents and one sister (aged 7 years). One male sibling, however, had died in the neonatal period in another hospital and from the history and postmortem findings it seems fairly certain that he died from a pneumothorax following inhalation of meconium as a result of foetal distress due to postmaturity. Another male sibling had been under our care with congenital heart disease and had died suddenly at home, aged seven months. The clinical diagnosis was VSD but this

Table I
Haematological investigations

[illegible]	Hb	Nucl. R.B.C. per mm.	Retic.	Platelets	Total W.B.C.	Neutrophils segm.	Neutrophils band	Neutrophils metamy. & myelo.	Eos.	Bas.	Mo.	L.	Smudge cells (per 100 W.B.C.)	Remarks
6.1	3.2	3.0	5.4		8,350	41	13			1	12	33	13	R.C.: Anisopoikil. Moderate polychromasia. Siderocytes + Rare histiocytes seen
10.12	3.4	3,900		3,000	5,200	18	2	1		1	9	69	7	Polychromasia ++ Siderocytes ++ R.B.C. 1.08 10⁶ cmm
15.1	11.1	1150	2.2	2,000	4,750	30	5	Transfusion			7	57	21	Siderocytes ++ 1 plasmacell
17.12	8.3	40		2,000	3,900	11	2		2	2	8	70	19	Siderocytes + 2 plasmacell
19.1	7.7				4,300	14	6				7	86	8	
22.12	7.3			2,000	5,300	9	1		2	1	3	83	2	1 plasmacell
27.12	11.1	1100			4,200	6	4	Transfusion			10	71	50	Siderocyte 6 plasmacell
2?.12	12	2 1??		3,000	7,4??								70	Siderocytes +

[illegible] small proportion of lymphocytes reminiscent of those seen in glandular fever

was before the days of regular cardiac catheterisation for such infants so the diagnosis was not confirmed.

On admission the patient was pyrexial (101 F) and showed extreme pallor profuse petechial purpura with ecchymoses and ankle oedema. There was no jaundice lymphadenopathy or hepato-splenomegaly.

Blood counts revealed pancytopenia (table I). The red cells showed anisopoikilocytosis, normochromia and slight polychromasia and few siderocytes were present, nucleated red cells were numerous the platelets were unequal in size. An occasional histiocytic monocyte and pair of endothelial cells (figs. 1 2 and 3) were seen in the first blood films but not on subsequent occasions number of smudged cells was also noted and small number of lymphocytes resembled those seen in glandular fever; there were no significant toxic changes in the neutrophils. Blood group was 0 Rh (D) positive. Direct Coombs test was negative. The serum looked normal, without trace of jaundice. Electrophoresis was normal and in particular showed no decrease of the albumin fraction. Blood cultures, throat swab and urine analysis gav normal results. Sternal puncture (December 7 1962) showed predominance of nucleated red cells. Erythropoiesis was normoblastic, slightly immature with an increased number of mitoses there ere few megaloid and binucleate cells. The leucon was represented mainly by immature cells (neutrophilic myelocytes), but no characteristically leukaemic forms were seen. The megakaryocytes were greatly reduced in number and no platelet formation was table. The plasmacells and tissue basophils were slightly increased. The striking feature as the presence of numerous macrophages (figs. 4—9) exhibiting mainly erythrophagocytosis. The tentative diagnosis of haemophagocytic reticulosis as made.

Prednisolone as gi en from 8th December 5 mg t.d.s. and from the 20th, 10 mg t.d.s. Sacral oedema was noticed on the 9th, some puffiness of the eyelids on the following day and oedema of the hands on the 14th. The same day he was transfused with 400 mls of blood which raised the haemoglobin to 11 1g%. There was rapid decline to 7 3 within eek—perhaps precipitated by repeat minor epistaxes—and the immediate improvement following the transfusion was not sustained. Pyrexia recurred after wo days and the liver edge as felt 3 fingerbreadths below the costal margin. A the same time (18th December) few cervical lymphnodes became palpable. He was transfused second time on the 26th December but haemorrhagic manifestations—ecchymoses on the pressure areas—became more pronounced on the 28th December. He died tw days later 25 days after admission.

Family studies. The parents were investigated haematologically and chromosomal analyses was carried out on skin biopsies by Dr Court Brown. The findings were normal throughout.

Post-mortem examination. The body was that of normally developed boy of three years ith generalised purpura. The liver appeared slightly enlarged and was somewhat brownish. The biliary system was normal. The spleen was enlarged (100 grams) and enclosed in bridged adhesions its cut surface showed several white and yellow infarcts. The tonsils, the mediastinal and mesenteric lymph glands ere enlarged and their cut surfaces were red. Sternal, ertebral and femoral marrow appeared macroscopically normal. The pericardium showed few ecchymoses and there was approximately 2 oz. of serous effusion. The lower lobes of the lungs felt firm. The kidney showed tiny cortical infarcts and the adrenals tiny haemorrhages. The stomach and intestines contained both altered and unaltered blood. The brain was not examined. No other abnormalities were noted.

Cytology

The pathognomonic feature of the bone-marrow smears was the presence of histiocytes with an amazing amount of phagocytosis. Histiocytes were especially nu-



3 4 5

Fig. Peripheral blood. Endothelial cells. 1,000.

Fig. Per... eral blood. Histiomonocyte. 1,000.

Fig. 3. Peripheral blood. ...onocyte with phagocytosed erythrocytes. ...

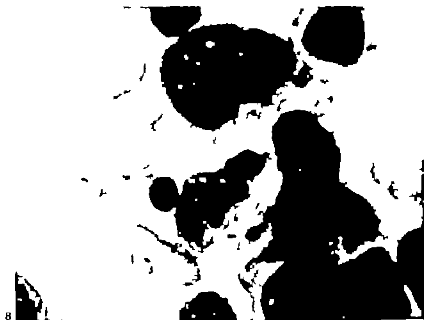
Fig. 4. Bone-marrow. Phagocytosed red cell and pigment granules. 1,...

Fig. 5. Bone-marrow. Epithelioid cell with two bean-shaped nuclei, ... cytoplasm and fringed borders. 1,...



Fig. 6. Bone-marrow. Three normoblasts and [illegible] least three erythrocytes in [illegible] single histiocyte. 1,500.

Fig. 7. Bone-marrow. Three normoblasts and several red cells in [illegible] binucleate reticulum cell. The re[illegible] bodies on the lower edge of the cell are parts of red cells possibly in the process of being phagocytosed. The metamyelocyte is perhaps overlying the macrophage. 1,500.



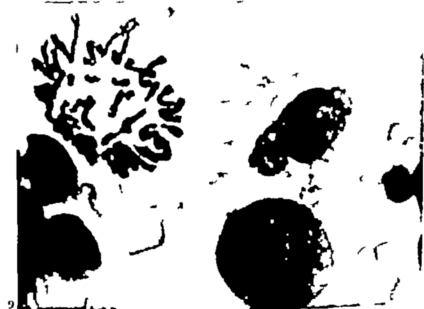


Fig 8 Bone-mar[illegible] [illegible] nevtual formation. Nucleoli, one larger and [illegible] largest nucleus [illegible]

Fig 9 Bone-marrow. An [illegible] reticular cell with large [illegible] [illegible] and macrophage [illegible]

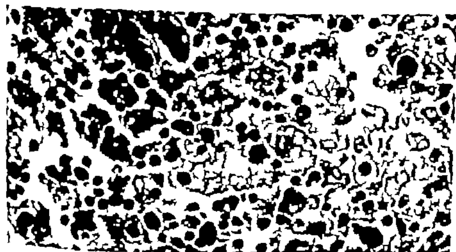


Fig 10 Erythrophagocytosis in mesenteric lymph node 550.

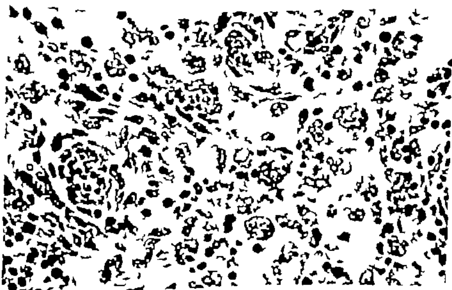


Fig. 11 Histiocytes in spleen containing erythrocytes. × 450.

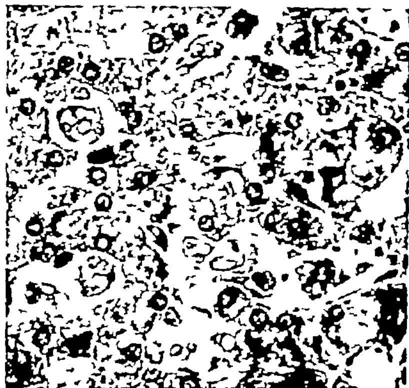


Fig. 1. Liver. Sinusoids containing macrophages with phagocytosed red cells. Some of the macrophages appear to be still in contact with the lining of the sinusoids. Kupffer cells. ×40.

merous in the 'tail' parts of the films. While some of the cells had well defined, though often irregular, fringed outlines (figs. 4, 5), others appeared to be 'torn' out of ... and lacked sharp borders and appeared to be derived from syncytial formations ... The former – those with sharp edges – were probably free histiocytes and the ... elements. The nuclei were either convoluted, 'monocytoid' or ... reminiscent of epithelioid cells as seen e.g. in tuberculosis, sarcoidosis or ... Some of the histiocytes had two or more nuclei, often of unequal size or in some cells, connected by single chromatin filaments. Their features apart, variation of the nuclear shape distinguished these cells ... seen in normal bone-marrow. The chromatin network was formed ... granular strands creating ... reticular nuclear pattern. Some ... two nucleoli. The cytoplasm was pale and often vacuolated. ... engulfed erythrocytes in various stages of lysis, nucleated red ... cells (mainly lymphocytes) ... in others only nuclear debris and granules which were Prussian blue negative. In addition to the early reticulum cells with ... nucleoli, often in the ... phages which probably were derived from them. Mitoses ... seen (fig. 9).

Histology

The essential feature was marked proliferation of reticuloendothelial cells with phagocytosis of erythrocytes and nucleated cells most pronounced in the spleen, lymph nodes, liver and bone-marrow

A mesenteric lymph node showed an essentially normal supporting architecture of capsule and trabecule the follicles were mainly small and some were distended from within by histiocytes. *In the medullary stroma and also in the cortex between the* follicles there was extensive histiocytic infiltration. Many of these reticuloendothelial cells showed phagocytosis, predominantly of red cells and also of brownish pigment (fig. 10) In mediastinal lymph node proliferation of histiocytes and phagocytosis was less pronounced. Minor scattered haemorrhages were seen.

The spleen showed loss of normal pattern due mainly to numerous haemorrhagic infarcts. Lymph follicles were well preserved in the non-infarcted areas. The pulp appeared hyperaemic, the network of spindle-shaped, fixed reticulum cells being clearly recognizable. Phagocytosis of red cells occurred mainly in the free histiocytes, large numbers of which were present in the sinusoids and ... (fig 11).

Sections of the liver showed marked widening of the sinusoids in which an amazing *amount of erythrophagocytosis by reticuloendothelial cells was taking place (fig. 12).* Most of these reticuloendothelial cells lay free in the sinusoids, but some were only partially detached and still preserved connection with the lining reticulum these cells were obviously derived from Kupffer cells, but appeared to have paler hypertrophic nuclei. The number of phagocytosed erythrocytes in single macrophage was quite remarkable. In addition to the distended sinusoids there were large pale foci of reticuloendothelial cells within the lobules, these foci having presumably resulted from more extensive proliferation of the reticuloendothelial cells in some of the sinusoids. Some of the macrophages contained brown pigment. The portal spaces were unremarkable.

The femoral bone-marrow was densely cellular normal elements being to great *extent replaced by sheets of histiocytes. Lymph follicles were numerous. Normal marrow* elements were represented mainly by nucleated red cells and myelocytes megakaryocytes were extremely sparse.

I the lungs there was extensive histiocytic infiltration of the interstitial tissue especially in the perivascular and peribronchial areas. Phagocytosis of red and white cells was noted in macrophages lying in the lumina of blood vessels. The kidney adrenals and heart muscle showed no histiocytic proliferations. There were several areas of necrosis in the myocardium and thrombus was seen in blood vessel supplying one of the necrotic areas.

Macrophages (or histiocytes) in spleen, lymph glands and bone-marrow (especially in the sinuses) contained large quantities of iron. Reticulin production was moderately *increased in the lymph glands and more so in the spleen.*

Discussion

The rapidly developing pallor and purpura in a severely ill, pyrexial patient suggested the diagnosis of acute leukaemia or possibly of septicaemia. The blood count, however failed to support either of these diagnoses as neither leukaemic cells nor septicaemic changes were seen. In the first peripheral film, after careful search, an occasional histiocyte and a pair of elongated

to two factors: dyshaemopoiesis and erythrophagocytosis. In the absence of pertinent investigations, i.e. red cell survival studies, we are unable to say whether the patient's red cells were of inferior quality or not. In view of the quick decline of the haemoglobin after transfusion, however, an enhanced extrinsic haemolytic *mechanism is very likely to be a major factor in the causation of the* anaemia. It is also very probable that the considerable degree of bone marrow replacement by histiocytes plays a not insignificant role in the development of pancytopenia. The presence of large numbers of smudge cells is an interesting feature and may have something to do with the presence of the phagocytosing histiocytes in the lymphoid tissues.

Acknowledgements: We wish to express our appreciation to Dr W. M. Court Brown, Medical Research Council, Clinical Effects of Radiation Research Unit, Western Infirmary, Edinburgh. We also wish to thank Mr J. M. Bevington, F.I.M.L.T. for the help with the photomicrographs.

Summary

A case of haemophagocytic reticulosis in a child of three years is described. The duration of the illness was five weeks and the course was clinically reminiscent of acute leukaemia. The diagnosis was made on the bone-marrow smears which showed large numbers of histiocytes phagocytosing red cells. These histiocytes not only destroy haematic cells by engulfing and digesting them, but almost certainly interfere with haemopoiesis also by their crowding out action and the consumption of essential metabolites. The value of an adequately cellular marrow aspirate is stressed as the only satisfactory mean of ante mortem diagnosis.

Résumé

Description d'un cas de réticulose hémophagocytaire chez un enfant de 3 ans. La maladie durait 5 semaines. L'évolution clinique rappelait une leucémie aiguë. Le diagnostic est établi sur les frottis de moelle osseuse qui contenait un grand nombre de phagocytes histiocytaires. Ces histiocytes détruisent non seulement des cellules sanguines, qu'ils résorbent et détruisent, mais entravent et détruisent probablement aussi l'hématopoèse par leur extension et par la consommation de métabolites essentiels. Les auteurs soulignent la valeur de l'aspiration d'une partie de la moelle suffisamment riche en cellules, comme seule méthode satisfaisante pour le diagnostic intravital.

Zusammenfassung

Es wird ein Fall von hämophagocytärer Retikulose bei einem dreijährigen Kind beschrieben. Die Krankheit dauerte 5 Wochen und der klinische Verlauf erinnerte an eine akute Leukämie. Die Diagnose wurde aus dem Knochenmarkausstrich gestellt, der eine große Zahl von phagocytierenden histiocytären Zellen aufwies. Diese Histiocyten zerstören nicht nur Blutzellen, die sie in sich aufnehmen und abbauen, sondern sehr wahrscheinlich beeinträchtigen sie auch die Blutzellbildung durch Überwucherung

Handbook of Physiology. Section 2: Circulation, Vol. II. Ed. by W. F. Hamilton. American Physiological Society, Washington, D. C. 1963. 1700 p., fig. and tab. Price US$ 32.—

Die American Physiological Society hat den Begriff, mit Hilfe einer grossen Zahl ausländiger Fachleute ein Handbuch über Physiologie herauszugeben und laufend, es durch periodische Revisionen am Leben zu erhalten. Früher erschien das Werk hierauf die erste Section »Neurophysiology«. Nun liegen 2 der 3 Volumina der zweiten Section »Circulation« vor. Der 1. Band umfasst die grundsätzlichen Aspekte des Kreislaufes. Im 2. Band, der soeben erschienen ist, werden Physiologie und Pathophysiologie der Gefässe und der Organ-Durchblutung dargestellt. Der 3. Band wird der Integration aller Kreislauffunktionen gewidmet sein. Es handelt sich ohne Zweifel um ein Werk von grossem Wert, das in jede naturwissenschaftliche und medizinische Bibliothek gehört.

W. Schweizer, Basel

W. S. Gleason, Jr., G. B. Highby and W. E. Kilgore: Atlas of Bone Tumors (including tumorlike lesions). C. V. Mosby, St. Louis 1963. 311 fig. (1-0 in color), Pr. $ 21.50.

The atlas of Dr. Gleason and associates contains X-ray reproductions and also colored photos of gross and histologic specimens obtained in different bone diseases. Unfortunately the quality of the majority of the colored photos of the gross specimens and the major part of the histological sections is questionable. The reproduction of the bone X-rays leaves even more to be desired.

In each chapter the photographic section is preceded by a short discussion of the disease.

Notwithstanding the weakness of the reproductions the text of the book is well worth studying, as it contains a number of excellent critical clinical remarks.

L. Saurer, Brooklyn, N. Y.

Charles E. Seiverd: Hematology for Medical Technologists. 3. ed. Lea & Febiger, Philadelphia 1964. 643 p., 180 illustr., 23 plc., $ 12.00.

The book has gained by changing its size and make-up since the 2. ed. was published (see Acta haemat., vol. 21, p. 320). A number of notes has been added; the detailed technical instructions show the experience and didactic ability of the author. More than 300 questions the technologists in U.S.A. have to answer when they take the examination for national registration, are listed in an appendix. The well printed book may serve as introduction into the daily haematologic technique. Most of the illustrations are good; the plate No. 13 is poor and should be replaced. O. Rosnow, New York

A. Buchert: Das Lupus erythematodes-Phänomen und die auslösenden Faktoren. G. Fischer Verlag, Jena 1963. 111 S., 12 Abb., 7 Tab., Preis DM 25.—

In diesem Büchlein wird eine kritische Übersicht zum Lupus erythematodes-Phänomen und den auslösenden Teilursachen gegeben. Der Autor konfrontiert seine eigenen Erfahrungen mit den Ansichten anderer Forscher. Die Ergebnisse der in vitro-Tests bei klinisch typischen Fällen von Lupus erythematodes werden denjenigen der verwandten Krankheiten wie primär chronischer Polyarthritis, Sklerodermie, Hydralazin-Syndrom und Lebererkrankungen (lupoide Hepatitis) gegenübergestellt. Buchert äussert sich sehr zurückhaltend zur Frage, ob bei Arzneimittelallergien un-

[illegible] Tumor: Microscopical and Centrifugation Studies on Living Cells») untersuchen [illegible] Tumoren: [illegible] physikalische Eigenschaften der Tumorzellen, die sich [illegible] in deren Zusammenhang [illegible] beobachten konnten, durch reduzierte [illegible] Kohäsion, besonders [illegible] Klebfähigkeit, geminderte Konsistenz und eine hohe Verformbarkeit bzw. Flexibilität [illegible]. Diese Eigenheiten der Tumorzellen werden als wichtige Kriterien für die Bildung von Tochtergeschwülsten bezeichnet. E. D. DAY («Vascular Relationships of Tumor and Host») befaßt sich mit der Frage der doppelten Gefäßversorgung eines Tumors, nämlich den Gefäßen im vorbestehenden Wirtsgewebe und denjenigen, welche der Tumor selbst als Teil seiner Stromaproliferation bildet. Es wird die Kenntnis dieser besonderen Gefäßverhältnisse als für die Chemotherapie eines Tumors bedeutungsvoll hervorgehoben. Die Problematik von Metastasierung und invasivem Wachstum heterologer Tumortransplantate wird von J. LEIGHTON («Invasion and Metastasis of Heterologous Tumors in the Chick Embryo») untersucht.

Das letzte große Kapitel befaßt sich mit der Carcinogenese. F. G. BOCK leitet es mit «Early Effects of Hydrocarbons on Mammalian Skin» ein. Die hier dargelegten Untersuchungen geben sehr interessante Hinweise bezüglich der Erfassung carcinogenbedingter Frühveränderungen. Diese Problematik der Früherfassung carcinogener Wirkungen hat eine hohe Aktualität. Folgerichtig schließt sich hier «Discussion on Cell Destruction and Population Dynamics in Experimental Skin Carcinogenesis in Mice» von O. H. [illegible] an. Auch in dieser Arbeit bilden die frühen Zellveränderungen den Hauptanliegen. Zur Frage der Bedeutung des [illegible] für die experimentelle Krebsforschung nimmt eine Untersuchung von R. K. BOUTWELL («Some Biological Aspects of Skin Carcinogenesis») Stellung. Dieser Themenkreis schließt mit einer Studie von H. CEMBER («Radiogenic Lung Cancer»), die das wie beim sogenannten «Schneeberger Lungenkrebs» bedeutsame Problem der Inhalation radioaktiven Materials ([illegible]) tierexperimentell erneut behandelt.

Betrachtet man rückblickend diesen neuen, 4. Band der «Fortschritte experimenteller Tumorforschung», so darf man wohl sagen, daß er vollauf seiner Aufgabe der zusammenfassenden Übersicht neuer Ergebnisse auf dem Gebiet der experimentellen Krebsforschung gerecht wird. Eine Fülle von Anregungen drängen sich dem Leser auf. Jeder, der sich experimentell oder praktisch mit den Problemen der Tumorforschung beschäftigt, wird nicht ohne Gewinn diesen Band aus der Hand legen.

W. MEIER-RUGE, Basel

S. Frankel and S. Reitman: *Gradwohl's Clinical Laboratory Methods and Diagnosis*, 6th ed. Mosby Co., Saint Louis 1963. Vol. 1: 1050 p.; Vol. 2: 1034 p., 754 fig., incl. 63 in color. Price $ 47.50.

After Dr. GRADWOHL's death, some years ago, 29 contributors worked on this new edition; with rigorous editing the contents of these volumes (over 2000 pages) could easily have been condensed considerably. Several chapters e.g. those on Electrocardiography do not belong in a book of this type.

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D. ROSENOW, New York

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O. ROSENOW, New York

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G. Rosenow, New York

2. Internationales Symposium für Chemotherapie. Part I: Neue Antibiotica, Nebenwirkungen der modernen Chemotherapeutica. Hgb. von P. Kuemmerle, P. Preziosi, P. Rentchnick. In: Antibiotica et Chemotherapia Vol. 11. Hgb. von O. Gsell. S. Karger Basel/New York 1963. XXII + 354 S., 64 Abb., 83 Tab., Preis sFr/DM 78.–.

Die Aufgabe, die Flut der am 2. Internationalen Symposium für Chemotherapie in Neapel 1961 gehaltenen Vorträge zugänglich zu machen, ist von Herausgeber und Verlag geschickt gelöst worden. Das Ergebnis ist ein 6bändiges Werk, das teils in der Reihe «Antibiotica et Chemotherapia», teils in der Zeitschrift «Chemotherapia» herausgekommen ist. Der erste Band enthält die Vorträge und Beiträge zu den Themen «Neue Antibiotica» und «Nebenwirkungen der modernen Chemotherapeutica» sowie die Panel-Discussion über *Laboratoriumstests* und ihre Anwendung in der täglichen Praxis der Antibiotica-Therapie.

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Die Panel-Discussion über die Laboratoriumstests und ihre praktische Anwendung in der täglichen Praxis der Antibiotica-Therapie leidet unter ihrer recht einseitigen Orientierung auf das Laboratorium hin. Weitere Beiträge befassen sich mit der sogenannten Substanz M, die offenbar zu den physiologischen Abwehrsubstanzen mit antituberkulöser Wirkung des tierischen und menschlichen Gewebes gehört, den *Mikamycinen, dem Actinoidin, dem Pimaricin, dem Trichomycin* und antibioticaproduzierenden Actinomyceten aus dem Abwasser. Die Nebenwirkungen der modernen Chemotherapeutica werden durch Gössner vom *Standpunkt* des Pathologen aus betrachtet, wobei vor allem auf den histochemischen Nachweis von Chemotherapeutica im Gewebsschnitt am Beispiel der Tetracycline und auf die Pathogenese cytotoxischer Effekte am Beispiel der Enzymhistochemie der Viomycinnephrose eingegangen wird. Dowling bespricht die Gründe für das zunehmende Auftreten von unerwünschten Nebenwirkungen bei Arzneimitteln mit antimikrobieller und antineoplastischer Wirkung und diskutiert die Indikationen für Hauttests zur Erfassung der Penicillinüberempfindlichkeit. Barrera zeigt, daß die sogenannten sekundären Wirkungen der antileukämischen Chemotherapeutica nicht als Nebenwirkungen aufgefaßt werden dürfen, sondern durch die gleiche Hauptwirkungsweise der Cytostatika bedingt sind.

H. Reber, Basel

Index rerum ad Vol. 31

Bearbeitet von G. Boehm, Basel

(B) = Buchbesprechungen – Livres nouveaux – Book reviews
(C) = Vorträge – Communications – Communications

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